



- (51) **International Patent Classification:**
C07K 14/195 (2006.01) *C12N 1/20* (2006.01)
C12P 7/46 (2006.01)
- (21) **International Application Number:** PCT/JP2015/086589
- (22) **International Filing Date:** 25 December 2015 (25.12.2015)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:** 2014-266560 26 December 2014 (26.12.2014) JP
- (71) **Applicant:** AJINOMOTO CO., INC. [JP/—]; 15-1, Kyobashi 1-chome, Chuo-ku, Tokyo, 1048315 (JP).
- (72) **Inventors:** HARA, Yoshihiko; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). FUKUI, Keita; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP). YAHAGI, Daiki; c/o AJINOMOTO CO., INC., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi, Kanagawa, 2108681 (JP).
- (74) **Agents:** KAWAGUCHI, Yoshiyuki et al.; Acropolis 21 Building 8th floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 1030004 (JP).

- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

(54) **Title:** METHOD FOR PRODUCING DICARBOXYLIC ACID

(57) **Abstract:** A method for producing a dicarboxylic acid is provided. A dicarboxylic acid is produced by culturing a bacterium having a dicarboxylic acid-producing ability, which has been modified so that the expression of one or more genes selected from *yeeA* gene, *ynfM* gene, *yjiP* gene, and *yjiB* gene is increased, in a medium, and collecting the dicarboxylic acid from the medium.



Description

Method for producing dicarboxylic acid

Technical Field

[0001]

The present invention relates to a method for producing a dicarboxylic acid using a bacterium.

Background Art

[0002]

Dicarboxylic acids such as succinic acid are produced by, for example, fermentation using a microorganism such as bacteria belonging to the family *Enterobacteriaceae* and coryneform bacteria. Specifically, there are known, for example, methods for producing a dicarboxylic acid such as succinic acid using a microorganism in which the expression of a gene encoding a transporter such as *sucE1* gene (Patent document 1) and *ybjL* gene (Patent document 2) has been enhanced.

Prior art references

Patent documents

[0003]

Patent document 1: WO2008/126896

Patent document 2: WO2008/133161

Summary of the Invention

Object to be Achieved by the Invention

[0004]

An object of the present invention is to develop a novel

2

technique for improving a dicarboxylic acid-producing ability of a bacterium and thereby provide a method for efficiently producing a dicarboxylic acid.

Means for Achieving the Object

[0005]

The inventors of the present invention conducted various researches in order to achieve the aforementioned object. As a result, they found *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene as genes encoding dicarboxylic acid efflux carriers, further found that dicarboxylic acid-producing ability of bacteria can be improved by modifying bacteria so that the expression of one or more of those genes is increased, and thus accomplished the present invention.

[0006]

That is, the present invention can be embodied, for example, as follows.

[1]

A method for producing a dicarboxylic acid, the method comprising:

culturing a bacterium having a dicarboxylic acid-producing ability in a medium to produce and accumulate the dicarboxylic acid in the medium; and

collecting the dicarboxylic acid from the medium,

wherein the bacterium has been modified so that the expression of one or more genes selected from *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene is increased.

[2]

The method mentioned above, wherein the expression of the gene(s) is increased by increasing the copy number of the

3

gene(s), and/or by modifying an expression control sequence of the gene(s).

[3]

The method mentioned above, wherein the *yeeA* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, or 6;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, or 6 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 2, 4, or 6, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, or 5;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 1, 3, or 5, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

[4]

The method mentioned above, wherein the *ynfM* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 8, 10, 12, 14, or 16;

(B) a DNA encoding a protein comprising the amino acid sequence

4

of SEQ ID NO: 8, 10, 12, 14, or 16 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;
(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 8, 10, 12, 14, or 16, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 7, 9, 11, 13, or 15;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 7, 9, 11, 13, or 15, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

[5]

The method mentioned above, wherein the *yjjP* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 18 or 20;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 18 or 20 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 18 or 20, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 17 or 19;

5

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 17 or 19, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

[6]

The method mentioned above, wherein the *yjjB* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 22 or 24;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 22 or 24 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 22 or 24, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 21 or 23;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 21 or 23, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

[7]

The method mentioned above, wherein the bacterium is a bacterium belonging to the family *Enterobacteriaceae*, or a coryneform bacterium.

6

[8]

The method mentioned above, wherein the bacterium belonging to the family *Enterobacteriaceae* is a *Pantoea* bacterium or an *Enterobacter* bacterium.

[9]

The method mentioned above, wherein the bacterium belonging to the family *Enterobacteriaceae* is *Pantoea ananatis* or *Enterobacter aerogenes*.

[10]

The method mentioned above, wherein the coryneform bacterium is a *Corynebacterium* bacterium.

[11]

The method mentioned above, wherein the coryneform bacterium is *Corynebacterium glutamicum*.

[12]

The method mentioned above, wherein the dicarboxylic acid consists of one or more dicarboxylic acids selected from the group consisting of α -ketoglutaric acid, malic acid, fumaric acid, succinic acid, and itaconic acid.

Brief Description of the Drawings

[0007]

[Fig. 1] Fig. 1 shows the results of itaconic acid production culture using an itaconic acid-producing bacterium, ITC01 strain (+CAD), and a control strain (-CAD). Fig. 1 (A) shows growth of the strains, and Fig. 1 (B) shows the accumulation amounts of itaconic acid.

Modes for Carrying out the Invention

[0008]

Hereafter, the present invention will be explained in detail.

[0009]

The method of the present invention is a method for producing a dicarboxylic acid, which comprises culturing a bacterium having a dicarboxylic acid-producing ability in a medium to produce and accumulate the dicarboxylic acid in the medium, and collecting the dicarboxylic acid from the medium, wherein the bacterium has been modified so that the expression of one or more genes selected from *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene is increased. The bacterium used for this method is also called "bacterium of the present invention". The *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene are also collectively referred to as "dicarboxylic acid efflux carrier gene".

[0010]

<1> Bacterium of the present invention

The bacterium of the present invention is a bacterium having a dicarboxylic acid-producing ability, which has been modified so that the expression of a dicarboxylic acid efflux carrier gene is increased.

[0011]

<1-1> Bacterium having dicarboxylic acid-producing ability

In the present invention, a "bacterium having a dicarboxylic acid-producing ability" refers to a bacterium having an ability to produce and accumulate an objective dicarboxylic acid in a medium in such a degree that the dicarboxylic acid can be collected, when the bacterium is cultured in the medium. The bacterium having a dicarboxylic acid-producing ability may be a bacterium that is able to

accumulate an objective dicarboxylic acid in a medium in an amount larger than that obtainable with a non-modified strain. The "non-modified strain" refers to a control strain that has not been modified so that the expression of a dicarboxylic acid efflux carrier gene is increased. Examples of the non-modified strain include wild strains and the parent strain of the bacterium. Specific examples of the non-modified strain include strains exemplified later, such as *Corynebacterium glutamicum* ATCC 13869 and ATCC 13032 strains for coryneform bacteria, and *Escherichia coli* K-12 W3110 (ATCC 27325) and MG1655 (ATCC 47076) strains, *Pantoea ananatis* SC17 (FERM BP-11091) and SC17(0) (VKPM B-9246) strains, and *Enterobacter aerogenes* AJ110637 strain (FERM BP-10955) for *Enterobacteriaceae* bacteria. The bacterium having a dicarboxylic acid-producing ability may be a bacterium that is able to accumulate an objective dicarboxylic acid in a medium in an amount of preferably 0.5 g/L or more, more preferably 1.0 g/L or more.

[0012]

Examples of the dicarboxylic acid include dicarboxylic acids having 3 to 8 carbon atoms (C_3 - C_8 dicarboxylic acids). Specific examples of the dicarboxylic acid include α -ketoglutaric acid (α -KG, synonymous with 2-oxoglutaric acid), malic acid, fumaric acid, succinic acid, itaconic acid, malonic acid, adipic acid, glutaric acid, pimelic acid, and suberic acid. The bacterium of the present invention may have an ability to produce only one kind of dicarboxylic acid, or may have an ability to produce two or more kinds of dicarboxylic acids.

[0013]

In the present invention, combination of the dicarboxylic

acid efflux carrier gene of which the expression is to be enhanced, and the dicarboxylic acid to be produced is not particularly limited. When the expression of the *yeeA* gene is enhanced, the dicarboxylic acid may be selected from, for example, α -KG, malic acid, fumaric acid, succinic acid, and itaconic acid. When the expression of the *ynfM* gene is enhanced, the dicarboxylic acid may be selected from, for example, α -KG, malic acid, fumaric acid, and succinic acid. When the expression of the *yjjPB* genes is enhanced, the dicarboxylic acid may be, for example, succinic acid.

[0014]

Examples of the bacterium include bacteria belonging to the family *Enterobacteriaceae* and coryneform bacteria.

[0015]

Examples of bacteria belonging to the family *Enterobacteriaceae* include bacteria belonging to the genus *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Photobacterium*, *Providencia*, *Salmonella*, *Morganella*, or the like. Specifically, bacteria classified into the family *Enterobacteriaceae* according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347>) can be used.

[0016]

The *Escherichia* bacteria are not particularly limited, and examples thereof include those classified into the genus *Escherichia* according to the taxonomy known to those skilled in the field of microbiology. Examples of the *Escherichia* bacteria include, for example, those described in the work of Neidhardt et al. (Backmann B.J., 1996, Derivations and

Genotypes of some mutant derivatives of *Escherichia coli* K-12, pp.2460-2488, Table 1, In F.D. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition, American Society for Microbiology Press, Washington, D.C.). Examples of the *Escherichia* bacteria include, for example, *Escherichia coli*. Specific examples of *Escherichia coli* include, for example, *Escherichia coli* K-12 strains such as W3110 strain (ATCC 27325) and MG1655 strain (ATCC 47076); *Escherichia coli* K5 strain (ATCC 23506); *Escherichia coli* B strains such as BL21 (DE3) strain; and derivative strains thereof.

[0017]

The *Enterobacter* bacteria are not particularly limited, and examples include those classified into the genus *Enterobacter* according to the taxonomy known to those skilled in the field of microbiology. Examples the *Enterobacter* bacterium include, for example, *Enterobacter agglomerans* and *Enterobacter aerogenes*. Specific examples of *Enterobacter agglomerans* include, for example, the *Enterobacter agglomerans* ATCC 12287 strain. Specific examples of *Enterobacter aerogenes* include, for example, the *Enterobacter aerogenes* ATCC 13048 strain, NBRC 12010 strain (Biotechnol. Bioeng., 2007, Mar. 27;98(2):340-348), and AJ110637 strain (FERM BP-10955). Examples the *Enterobacter* bacteria also include, for example, the strains described in European Patent Application Laid-open (EP-A) No. 0952221. In addition, *Enterobacter agglomerans* also include some strains classified as *Pantoea agglomerans*.

[0018]

The *Pantoea* bacteria are not particularly limited, and examples include those classified into the genus *Pantoea*

according to the taxonomy known to those skilled in the field of microbiology. Examples the *Pantoea* bacteria include, for example, *Pantoea ananatis*, *Pantoea stewartii*, *Pantoea agglomerans*, and *Pantoea citrea*. Specific examples of *Pantoea ananatis* include, for example, the *Pantoea ananatis* LMG20103 strain, AJ13355 strain (FERM BP-6614), AJ13356 strain (FERM BP-6615), AJ13601 strain (FERM BP-7207), SC17 strain (FERM BP-11091), SC17(0) strain (VKPM B-9246), and SC17sucA strain (FERM BP-8646). Some of *Enterobacter* bacteria and *Erwinia* bacteria were reclassified into the genus *Pantoea* (Int. J. Syst. Bacteriol., 39, 337-345 (1989); Int. J. Syst. Bacteriol., 43, 162-173 (1993)). For example, some strains of *Enterobacter agglomerans* were recently reclassified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii*, or the like on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Bacteriol., 39, 337-345 (1989)). In the present invention, the *Pantoea* bacteria include those reclassified into the genus *Pantoea* as described above.

[0019]

Examples of the *Erwinia* bacteria include *Erwinia amylovora* and *Erwinia carotovora*. Examples of the *Klebsiella* bacteria include *Klebsiella planticola*.

[0020]

Examples of the coryneform bacteria include bacteria belonging to the genus *Corynebacterium*, *Brevibacterium*, *Microbacterium*, or the like.

[0021]

Specific examples of the coryneform bacteria include the following species.

Corynebacterium acetoacidophilum

12

Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium crenatum
Corynebacterium glutamicum
Corynebacterium lilium
Corynebacterium melassecola
Corynebacterium thermoaminogenes (*Corynebacterium efficiens*)
Corynebacterium herculis
Brevibacterium divaricatum (*Corynebacterium glutamicum*)
Brevibacterium flavum (*Corynebacterium glutamicum*)
Brevibacterium immariophilum
Brevibacterium lactofermentum (*Corynebacterium glutamicum*)
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Corynebacterium ammoniagenes (*Corynebacterium stationis*)
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum

[0022]

Specific examples of the coryneform bacteria include the following strains.

Corynebacterium acetoacidophilum ATCC 13870
Corynebacterium acetoglutamicum ATCC 15806
Corynebacterium alkanolyticum ATCC 21511
Corynebacterium callunae ATCC 15991

13

Corynebacterium crenatum AS1.542

Corynebacterium glutamicum ATCC 13020, ATCC 13032, ATCC 13060, ATCC 13869, FERM BP-734

Corynebacterium lilium ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium efficiens (*Corynebacterium thermoaminogenes*) AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC 13868

Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC 14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205)

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Corynebacterium ammoniagenes (*Corynebacterium stationis*) ATCC 6871, ATCC 6872

Brevibacterium album ATCC 15111

Brevibacterium cerinum ATCC 15112

Microbacterium ammoniaphilum ATCC 15354

[0023]

The *Corynebacterium* bacteria include bacteria that had previously been classified into the genus *Brevibacterium*, but are presently united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1991)). Moreover, *Corynebacterium stationis* includes bacteria that had previously been classified as *Corynebacterium ammoniagenes*, but are presently

re-classified into *Corynebacterium stationis* on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Evol. Microbiol., 60, 874-879 (2010)).

[0024]

These strains are available from, for example, the American Type Culture Collection (Address: 12301 Parklawn Drive, Rockville, Maryland 20852, P.O. Box 1549, Manassas, VA 20108, United States of America). That is, registration numbers are given to the respective strains, and the strains can be ordered by using these registration numbers (refer to <http://www.atcc.org/>). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection. These strains can also be obtained from, for example, the depositories at which the strains were deposited.

[0025]

The bacterium of the present invention may be a bacterium inherently having a dicarboxylic acid-producing ability, or may be a bacterium modified so that it has a dicarboxylic acid-producing ability. The bacterium having a dicarboxylic acid-producing ability can be obtained by imparting a dicarboxylic acid-producing ability to such a bacterium as mentioned above, or enhancing a dicarboxylic acid-producing ability of such a bacterium as mentioned above.

[0026]

Hereafter, specific examples of the dicarboxylic acid-producing bacteria and the method for imparting or enhancing a dicarboxylic acid-producing ability will be explained. Such modifications as exemplified below for imparting or enhancing a dicarboxylic acid-producing ability may be independently used, and may be used in an appropriate

combination.

[0027]

Examples of the method for imparting or enhancing a dicarboxylic acid-producing ability include a method of modifying a bacterium so that the activity of an enzyme that catalyzes a reaction branching off from the biosynthesis pathway of an objective dicarboxylic acid to generate a compound other than the objective dicarboxylic acid is reduced. The "enzyme that catalyzes a reaction branching off from the biosynthesis pathway of an objective dicarboxylic acid to generate a compound other than the objective dicarboxylic acid" referred to here also includes an enzyme involved in decomposition of the objective dicarboxylic acid. The activity or activities of one or two or more kinds of enzymes may be reduced. An enzyme activity can be reduced, for example, by reducing the expression of a gene encoding the enzyme, or by disrupting a gene encoding the enzyme as described later.

[0028]

For example, succinic acid-producing ability can be imparted or enhanced by reducing the activity or activities of one or more enzymes of the lactic acid biosynthesis system (WO2005/052135, WO2005/116227, U.S. Patent No. 5,770,435, U.S. Patent Published Application No. 20070054387, WO99/53035, Alam, K.Y. and Clark, D.P., 1989, J. Bacteriol., 171:6213-6217). The same shall apply to the other dicarboxylic acids such as α -KG, malic acid, fumaric acid, and itaconic acid. Examples of the enzymes of the lactic acid biosynthesis system include lactate dehydrogenase (*ldhA*). The nucleotide sequence of the *ldhA* gene of the *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 101, and the amino acid sequence of the protein encoded by this

gene is shown as SEQ ID NO: 102. Shown in the parenthesis following the enzyme name is an example of gene encoding the enzyme (the same shall apply to the following descriptions).
[0029]

The "lactate dehydrogenase" refers to a protein having an activity for catalyzing a reaction of generating lactate from pyruvate using NADH or NADPH as an electron donor. This activity may also be referred to as "lactate dehydrogenase activity". The lactate dehydrogenase is roughly classified into L-lactate dehydrogenase (L-LDH, EC 1.1.1.27) that generates L-lactic acid, and D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) that generates D-lactate, and the activity or activities of either or both of them may be reduced. Reduction of the lactate dehydrogenase activity can be confirmed by, for example, measuring the lactate dehydrogenase activity by a known method (L. Kanarek and R.L. Hill, J. Biol. Chem., 239, 4202 (1964)). Specific examples of the method for constructing a mutant strain of *Enterobacteriaceae* bacterium having a reduced lactate dehydrogenase activity include the method described in Alam, K.Y., Clark, D.P., 1989, J. Bacteriol., 171, 6213-6217, and so forth.

[0030]

Succinic acid-producing ability can also be imparted or enhanced by reducing the activity or activities of one or more enzymes of the acetic acid biosynthesis system (U.S. Patent Published Application No. 20070054387, WO2005/052135, WO99/53035, WO2006/031424, WO2005/113745, WO2005/113744). The same shall apply to the other dicarboxylic acids such as α -KG, malic acid, fumaric acid, and itaconic acid. Examples of the enzymes of the acetic acid biosynthesis system include

phosphotransacetylase (*pta*), acetate kinase (*ack*), pyruvate oxidase (*poxB*), acetyl-CoA synthetase (*acs*), and acetyl-CoA hydrolase.

[0031]

"Phosphotransacetylase" refers to a protein having an activity for catalyzing a reaction of generating CoA and acetyl phosphate from acetyl-CoA and phosphate (EC 2.3.1.8). This activity may also be referred to as "phosphotransacetylase activity". Reduction of the phosphotransacetylase activity can be confirmed by measuring the phosphotransacetylase activity by a known method (Klotzsch, H.R., Meth. Enzymol., 12, 381-386 (1969)).

[0032]

Succinic acid-producing ability can also be imparted or enhanced by reducing the activity or activities of one or more enzymes of the formic acid biosynthesis system (U.S. Patent Published Application No. 20070054387, WO2005/116227, WO2005/52135, Donnelly, M.I. et al., 1998, Appl. Biochem. Biotechnol., 70-72:187-198). The same shall apply to the other dicarboxylic acids such as α -KG, malic acid, fumaric acid, and itaconic acid. Examples of the enzymes of the formic acid biosynthesis system include pyruvate formate lyase (*pflB*, *pflD*, *tdcE*).

[0033]

"Pyruvate formate lyase" refers to a protein having an activity for catalyzing a reaction of generating acetyl-CoA and formate from pyruvate and CoA (EC 2.3.1.54). This activity may also be referred to as "pyruvate formate lyase activity". Reduction of the pyruvate formate lyase activity can be confirmed by measuring the pyruvate formate lyase activity by

a known method (Knappe, J. & Blaschkowski, H.P., Meth. Enzymol., 41, 508-518 (1975)).

[0034]

Succinic acid-producing ability can also be imparted or enhanced by reducing the activity or activities of one or more enzymes of the ethanol biosynthesis system (WO2006/031424). The same shall apply to the other dicarboxylic acids such as α -KG, malic acid, fumaric acid, and itaconic acid. Examples of the enzymes of the ethanol biosynthesis system include alcohol dehydrogenase (*adhE*). The nucleotide sequence of the *adhE* gene of the *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 103, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 104.

[0035]

"Alcohol dehydrogenase" refers to a protein having an activity for catalyzing a reaction of generating an alcohol from an aldehyde by using NADH or NADPH as an electron donor (EC 1.1.1.1, EC 1.1.1.2, or EC 1.1.1.71). This activity may also be referred to as "alcohol dehydrogenase activity". Reduction of the alcohol dehydrogenase activity can be confirmed by, for example, measuring the alcohol dehydrogenase activity by a known method (Lutstorf, U.M., Schurch, P.M. & von Wartburg, J.P., Eur. J. Biochem., 17, 497-508 (1970)). Specific examples of the method for constructing a mutant strain of *Enterobacteriaceae* bacterium having a reduced alcohol dehydrogenase activity include the method described in Sanchez, A.M., Bennett, G.N., San, K-Y., Biotechnol. Prog., 21, 358-365 (2005), and so forth.

[0036]

Succinic acid-producing ability can also be imparted or

enhanced by reducing the activity or activities of one or more enzymes of the 2,3-butanediol biosynthesis system. The same shall apply to the other dicarboxylic acids such as α -KG, malic acid, fumaric acid, and itaconic acid. Examples of the enzymes of the 2,3-butanediol biosynthesis system include acetolactate synthase (*budB*, *ilvB*, *ilvG*, *ilvI*), acetolactate decarboxylase (*budA*), and acetoin reductase (*budC*, *butA*). The nucleotide sequences of *budB*, *budA*, and *budC* genes of the *Pantoea ananatis* AJ13355 strain are shown as SEQ ID NOS: 105, 107, and 109, respectively, and the amino acid sequences of the proteins encoded by these genes are shown as SEQ ID NOS: 106, 108, and 110, respectively.

[0037]

"Acetolactate synthase" refers to a protein having an activity for catalyzing a reaction of generating acetolactate and CO₂ from two molecules of pyruvate (EC 2.2.1.6). This activity may also be referred to as "acetolactate synthase activity". The isozymes AHAS I to III are known for the acetolactate synthase (AHAS), and the activity or activities of any one or more of these isozymes may be reduced. Reduction of the acetolactate synthase activity can be confirmed by, for example, measuring the acetolactate synthase activity by a known method (F.C. Stormer and H.E. Umbarger, Biochem. Biophys. Res. Commun., 17, 5, 587-592 (1964)).

[0038]

"Acetolactate decarboxylase" refers to a protein having an activity for catalyzing a reaction of decarboxylating acetolactate to generate acetoin (EC 4.1.1.5). This activity may also be referred to as "acetolactate decarboxylase activity". For example, *E. coli* and *Corynebacterium*

glutamicum do not have the acetolactate decarboxylase. Reduction of the acetolactate decarboxylase activity can be confirmed by, for example, measuring the acetolactate decarboxylase activity by a known method (Juni E., J. Biol. Chem., 195(2):715-726 (1952)).

[0039]

"Acetoin reductase" refers to a protein having an activity for catalyzing a reaction of generating 2,3-butanediol from acetoin using NADH or NADPH as an electron donor (EC 1.1.1.4). This activity may also be referred to as "acetoin reductase activity". For example, *E. coli* does not have the acetoin reductase. Reduction of the acetoin reductase activity can be confirmed by, for example, measuring the acetoin reductase activity by a known method (K. Blomqvist et al., J. Bacteriol., 175, 5, 1392-1404 (1993)).

[0040]

Succinic acid-producing ability can also be imparted or enhanced by reducing the activity or activities of one or more enzymes selected from pyruvate kinase (*pykF*, *pykA*), glucose PTS (*ptsG*), ArcA protein (*arcA*), IclR protein (*iclR*), glutamate dehydrogenase (*gdhA*), glutamine synthetase (*glnA*), and glutamate synthase (*gltBD*) (WO2006/107127, WO2007/07933, Japanese Patent Laid-open (Kokai) No. 2005-168401). The succinic acid-producing ability can also be imparted or enhanced by reducing the activity of succinate dehydrogenase (*sdhA*). The nucleotide sequence of the *sdhA* gene of the *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 111, and the amino acid sequence of the protein encoded by the gene is shown as SEQ ID NO: 112.

[0041]

21

"Succinate dehydrogenase" refers to a protein having an activity for catalyzing a reaction of oxidizing succinic acid using quinone as an electron acceptor (EC 1.3.5.1). This activity may also be referred to as "succinate dehydrogenase". Reduction of the succinate dehydrogenase activity can be confirmed by, for example, measuring the succinate dehydrogenase activity by a known method (Tatsuki Kurokawa and Junshi Sakamoto, Arch. Microbiol., 183:317-324 (2005)).

[0042]

Malic acid-producing ability can be imparted or enhanced by reducing the activity or activities of one or more enzymes selected from malate dehydrogenase (*mdh*), malate-quinone oxidoreductase (*mgo*), and malic enzyme (*sfcA*, *maeB*). The nucleotide sequences of *mdh*, *mgo1*, *mgo2*, *sfcA*, and *maeB* genes of the *Pantoea ananatis* AJ13355 strain are shown as SEQ ID NOS: 113, 115, 117, 119, and 121, respectively, and the amino acid sequences of the proteins encoded by these genes are shown as SEQ ID NOS: 114, 116, 118, 120, and 122, respectively.

[0043]

"Malate-quinone oxidoreductase" refers to a protein having an activity for catalyzing a reaction of oxidizing malate by using quinone as an electron acceptor. This activity may also be referred to as "malate-quinone oxidoreductase activity". The malate-quinone oxidoreductase conjugates with the NAD-type malate dehydrogenase to form a cycle of malate and oxaloacetate, and thereby provide net oxidation of NADH. For example, some of *Pantoea* bacteria have 2 copies of the malate-quinone oxidoreductase gene. In such a case, either one of the genes may be disrupted or the like, or the both genes may be disrupted or the like. Reduction of the malate-quinone oxidoreductase

activity can be confirmed by, for example, measuring the malate-quinone oxidoreductase activity by a known method (Hoyt J.C. et al. (1988) Biochim. Biophys. Acta, 14;966(1):30-5; Mackintosh, C. et al. (1988) Biochem. J., 250, 25-31).

[0044]

Itaconic acid-producing ability can be imparted or enhanced by reducing the activity or activities of one or more enzymes selected from isocitrate dehydrogenase (*icd*) and isocitrate lyase (*aceA*).

[0045]

"Isocitrate lyase" refers to a protein having an activity for reversibly catalyzing a reaction of generating glyoxylate and succinate from isocitrate (EC 4.1.3.1). This activity may also be referred to as "isocitrate lyase activity". For example, some of *Corynebacterium* bacteria have 2 copies of the isocitrate lyase gene. In such a case, either one of the genes may be disrupted or the like, or the both genes may be disrupted or the like. Reduction of the isocitrate lyase activity can be confirmed by, for example, measuring the isocitrate lyase activity by a known method (Hoyt J.C. et al. (1988) Biochim. Biophys. Acta, 14;966(1):30-5; Mackintosh, C. et al. (1988) Biochem. J., 250, 25-31)

[0046]

The *aceA* gene encoding isocitrate lyase typically forms an operon consisting of the *aceBAK* genes. The *aceB* gene is a gene encoding malate synthase. The *aceK* gene is a gene encoding isocitrate dehydrogenase kinase/phosphatase. For reducing the isocitrate lyase activity, the whole of the *aceBAK* genes may also be disrupted or the like. The nucleotide sequences of the *aceB*, *aceA*, and *aceK* genes of the *Pantoea ananatis* AJ13355

23

strain are shown as SEQ ID NOS: 123, 125, and 127, respectively, and the amino acid sequences of the proteins encoded by these genes are shown as SEQ ID NOS: 124, 126, and 128, respectively. [0047]

Itaconic acid-producing ability can also be imparted or enhanced by increasing the activity of cis-aconitate decarboxylase (CAD). Cis-aconitate decarboxylase (CAD) refers to a protein having an activity for catalyzing a reaction of decarboxylating cis-aconitate to generate itaconate (EC 4.1.1.6). This activity may also be referred to as "CAD activity". Increase of CAD activity can be confirmed by, for example, measuring CAD activity by a known method (L. Dwiarti, K. Yamane, H. Yamatani, P. Kahar, M. Okabe. Purification and characterization of cis-aconitic acid decarboxylase from *Aspergillus terreus* TN484-M1. J. of Bioscience and Bioengineering, 94(1), p.29-33, 2004). Examples of the cis-aconitate decarboxylase gene (CAD gene) include the CAD gene of *Aspergillus terreus* (Japanese Patent Laid-open (Kokai) No. 2013-051900). The nucleotide sequence of the CAD gene of *Aspergillus terreus* optimized for the codon usage of *E. coli* is shown as SEQ ID NO: 49. In the nucleotide sequence of SEQ ID NO: 49, the coding region corresponds to the positions 91 to 1563. The amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 131.

[0048]

α -KG-producing ability can be imparted or enhanced by reducing the activity of α -ketoglutarate dehydrogenase (*sucA*, *odhA*). The nucleotide sequence of the *sucA* gene of *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 129, and the amino acid sequence of the protein encoded by this gene is shown as

SEQ ID NO: 130.

[0049]

Examples of the method for imparting or enhancing a dicarboxylic acid-producing ability also include a method of modifying a bacterium so that the activity of an enzyme of the biosynthesis system of the objective dicarboxylic acid is increased. The activity or activities of one or two or more kinds of enzymes may be increased. An enzyme activity can be increased by, for example, enhancing the expression of a gene encoding the enzyme as described later.

[0050]

For example, a dicarboxylic acid-producing ability can be imparted or enhanced by increasing the activity or activities of one or more enzymes of an anaplerotic pathway of the TCA cycle. Examples of the enzymes of an anaplerotic pathway of the TCA cycle include pyruvate carboxylase (*pyc*), phosphoenolpyruvate carboxylase (*ppc*), phosphoenolpyruvate carboxykinase (*pckA*), citrate synthase (*gltA*), and methyl citrate synthase (*prpC*).

[0051]

Examples of genes encoding pyruvate carboxylase include, for example, *pyc* genes of coryneform bacteria such as *Corynebacterium glutamicum* and *Brevibacterium flavum*, *Bacillus stearothermophilus*, *Rhizobium etli*, and yeast such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (WO2009/072562). Examples of genes encoding phosphoenolpyruvate carboxykinase include, for example, *pckA* gene of *Actinobacillus succinogenes* (GenBank Accession No. YP_001343536.1), *pckA* gene of *Haemophilus influenzae* (GenBank Accession No. YP_248516.1), *pckA* gene of *Pasteurella multocida* (GenBank Accession No. NP_246481.1), *pckA* gene of *Mannheimia*

succiniciproducens (GenBank Accession No. YP_089485.1), *pckA* gene of *Yersinia pseudotuberculosis* (GenBank Accession No. YP_072243), *pckA* gene of *Vibrio cholerae* (GenBank Accession No. ZP_01981004.1), and *pckA* gene of *Selenomonas ruminantium* (GenBank Accession No. AB016600) (WO2009/072562). Examples of genes encoding phosphoenolpyruvate carboxylase include, for example, *ppc* genes of coryneform bacteria such as *Corynebacterium glutamicum* and *Brevibacterium flavum*, *Escherichia* bacteria such as *Escherichia coli*, and *Rhodopseudomonas palustris*. An enzyme activity can also be increased by, for example, reducing or eliminating feedback inhibition. For example, the activity of phosphoenolpyruvate carboxylase (PEPC) is inhibited by L-malic acid, which is an intermediate product of the succinic acid biosynthesis pathway (Masato Yano and Katsura Izui, Eur. Biochem. FEBS, 247, 74-81, 1997). The inhibition by L-malic acid can be reduced by, for example, introducing a desensitization mutation based on one amino acid substitution into PEPC. Specific example of the desensitization mutation based on one amino acid substitution include, for example, a mutation for replacing the 620th amino acid, lysine, with serine, in the PEPC protein of *Escherichia coli* (ibid.).

[0052]

Examples of dicarboxylic acid-producing bacteria include the bacteria described in Fermentation Handbook (Kyoritsu Shuppan).

[0053]

Specific examples of succinic acid-producing bacteria belonging to the family *Enterobacteriaceae* include the following strains.

26

Escherichia coli SS373 strain (WO99/06532)

Escherichia coli AFP111 strain (WO97/6528)

Escherichia coli NZN111 strain (U.S. Patent No. 6,159,738)

Escherichia coli AFP184 strain (WO2005/116227)

Escherichia coli SBS100MG strain, SBS110MG strain, SBS440MG strain, SBS550MG strain, and SBS660MG strain (WO2006/031424)

Enterobacter aerogenes AJ110637 strain (FERM BP-10955)

Enterobacter aerogenes VP-1 strain (J. Biosci. Bioeng., 2004, 97(4):227-32)

[0054]

Specific examples of succinic acid-producing bacteria belonging to coryneform bacteria include the following strains.

Brevibacterium flavum AB-41 strain (Japanese Patent Laid-open (Kokai) No. 11-113588)

Brevibacterium flavum PC-amplified strain of AB-41 strain (Japanese Patent Laid-open (Kokai) No. 11-196888)

Corynebacterium glutamicum AJ110655 strain (FERM BP-10951)

Brevibacterium flavum MJ233 Δ ldh strain (WO2005/021770)

Brevibacterium lactofermentum 2256 Δ (ldh,ach,pta,ack) (WO2005/113744)

Brevibacterium lactofermentum 2256 Δ (ldh,pta,ack,poxB) (WO2005/113745)

Corynebacterium glutamicum R ldh-/pCRB-1 PC strain (WO2005/010182)

[0055]

The genes and proteins used for breeding dicarboxylic acid-producing bacteria may have, for example, the nucleotide sequences and amino acid sequences of known genes and proteins, such as the nucleotide sequences and amino acid sequences exemplified above, respectively. Also, the genes and proteins

used for breeding dicarboxylic acid-producing bacteria may be conservative variants of known genes and proteins, such as conservative variants of genes and proteins having the nucleotide sequences and amino acid sequences exemplified above, respectively. Specifically, for example, the genes used for breeding dicarboxylic acid-producing bacteria may each be a gene encoding a protein having an amino acid sequence of a known protein including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions. As for conservative variants of genes or proteins, the descriptions concerning conservative variants of the dicarboxylic acid efflux carrier gene and dicarboxylic acid efflux carrier mentioned later can be applied *mutatis mutandis*.
[0056]

<1-2> Enhancement of expression of dicarboxylic acid efflux carrier gene

The bacterium of the present invention has been modified so that the expression of a dicarboxylic acid efflux carrier gene is increased. A dicarboxylic acid-producing ability of a bacterium can be improved by modifying the bacterium so that the expression of a dicarboxylic acid efflux carrier gene is increased.

[0057]

The bacterium of the present invention can be obtained by modifying a bacterium having a dicarboxylic acid-producing ability so that the expression of a dicarboxylic acid efflux carrier gene is increased. The bacterium of the present invention can also be obtained by modifying a bacterium so that the expression of a dicarboxylic acid efflux carrier gene is increased, and then imparting dicarboxylic acid-producing

ability or enhancing a dicarboxylic acid-producing ability thereof. The bacterium of the present invention may also be a bacterium that has been acquired a dicarboxylic acid-producing ability by being modified so that the expression of a dicarboxylic acid efflux carrier gene is increased. The modifications for constructing the bacterium of the present invention can be performed in an arbitrary order.

[0058]

The dicarboxylic acid efflux carrier gene is selected from the *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene. Although it has not been known that these genes are dicarboxylic acid efflux carrier genes, it is estimated that these genes are dicarboxylic acid efflux carrier genes on the basis of the results of the experiments shown in the Examples section of this description. The proteins encoded for by the *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene are also referred to as YeeA protein, YnfM protein, YjjP protein, and YjjB protein, respectively. In the present invention, the expression of one kind of dicarboxylic acid efflux carrier gene may be enhanced, or the expression of two or more kinds of dicarboxylic acid efflux carrier genes may be enhanced. For example, the expression of the *yjjP* gene and *yjjB* gene may be enhanced.

[0059]

<*yeeA* gene>

The *yeeA* gene is a gene encoding a protein presumed to be a conserved inner membrane protein. The *yeeA* gene of the *Escherichia coli* MG1655 strain is also referred to as *b2008* or *ECK2002*. The nucleotide sequence of the *yeeA* gene of the *Escherichia coli* MG1655 strain is shown as SEQ ID NO: 1, and the amino acid sequence of the protein encoded by this gene

(GenBank Accession No. NP_416512.1) is shown as SEQ ID NO: 2. The nucleotide sequence of the *yeeA* gene of the *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 3, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 4. The nucleotide sequence of the *yeeA* gene of the *Enterobacter aerogenes* AJ110637 strain is shown as SEQ ID NO: 5, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 6.

[0060]

<*ynfM* gene>

The *ynfM* gene is a gene encoding a protein presumed to be a predicted transport protein YnfM. The *ynfM* gene of the *Escherichia coli* MG1655 strain is also referred to as *b1596* or *ECK1591*. The nucleotide sequence of the *ynfM* gene of the *Escherichia coli* MG1655 strain is shown as SEQ ID NO: 7, and the amino acid sequence of the protein encoded by this gene (GenBank Accession No. NP_416113.1) is shown as SEQ ID NO: 8. The nucleotide sequence of the *ynfM* gene of *Pantoea ananatis* AJ13355 strain is shown as SEQ ID NO: 9, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 10. The nucleotide sequence of the *ynfM* gene of the *Enterobacter aerogenes* AJ110637 strain is shown as SEQ ID NO: 11, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 12. The nucleotide sequence of the *ynfM* gene of *Corynebacterium glutamicum* ATCC 13032 is shown as SEQ ID NO: 13, and the amino acid sequence of the protein encoded by this gene (GenBank Accession No. NP_602116.1) is shown as SEQ ID NO: 14. The nucleotide sequence of the *ynfM* gene of *Corynebacterium glutamicum* ATCC 13869 is shown as SEQ ID NO: 15, and the amino acid sequence of the protein encoded by this

gene is shown as SEQ ID NO: 16.

[0061]

<yjjP gene>

The *yjjP* gene is a gene encoding a protein presumed to be a predicted inner membrane structural protein. The *yjjP* gene of the *Escherichia coli* MG1655 strain is also referred to as *b4364* or *ECK4354*. The nucleotide sequence of the *yjjP* gene of the *Escherichia coli* MG1655 strain is shown as SEQ ID NO: 17, and the amino acid sequence of the protein encoded by this gene (GenBank Accession No. NP_418784.4) is shown as SEQ ID NO: 18. The nucleotide sequence of the *yjjP* gene of the *Enterobacter aerogenes* AJ110637 strain is shown as SEQ ID NO: 19, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 20.

[0062]

<yjjB gene>

The *yjjB* gene is a gene encoding a protein presumed to be a conserved inner membrane protein. The *yjjB* gene of the *Escherichia coli* MG1655 strain is also referred to as *b3463* or *ECK4353*. The nucleotide sequence of the *yjjB* gene of the *Escherichia coli* MG1655 strain is shown as SEQ ID NO: 21, and the amino acid sequence of the protein encoded by this gene (GenBank Accession No. NP_418783.2) is shown as SEQ ID NO: 22. The nucleotide sequence of the *yjjB* gene of the *Enterobacter aerogenes* AJ110637 strain is shown as SEQ ID NO: 23, and the amino acid sequence of the protein encoded by this gene is shown as SEQ ID NO: 24.

[0063]

That is, the dicarboxylic acid efflux carrier gene may be, for example, a gene (such as a DNA) having the nucleotide

sequence shown as SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23. Also, the dicarboxylic acid efflux carrier may be, for example, a protein having the amino acid sequence shown as SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24. The expression "a gene or protein has a nucleotide or amino acid sequence" encompasses cases where a gene or protein comprises the nucleotide or amino acid sequence, and cases where a gene or protein consists of the nucleotide or amino acid sequence. [0064]

The dicarboxylic acid efflux carrier gene may be a variant of the dicarboxylic acid efflux carrier genes exemplified above (namely, *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene exemplified above), so long as the original function thereof is maintained. Similarly, the dicarboxylic acid efflux carrier may be a variant of the dicarboxylic acid efflux carriers exemplified above (namely, YeeA protein, YnfM protein, YjjP protein, and YjjB protein above-mentioned), so long as the original function thereof is maintained. Such a variant that maintains the original function may also be referred to as "conservative variant". That is, the terms "*yeeA* gene", "*ynfM* gene", "*yjjP* gene", and "*yjjB* gene" include not only the *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene exemplified above, respectively, but also include conservative variants thereof. Similarly, the terms "YeeA protein", "YnfM protein", "YjjP protein", and "YjjB protein" include not only the YeeA protein, YnfM protein, YjjP protein, and YjjB protein exemplified above, respectively, but also include conservative variants thereof. Examples of the conservative variant include, for example, homologues and artificially modified versions of the dicarboxylic acid efflux carrier genes and dicarboxylic acid

efflux carriers exemplified above.

[0065]

The expression that "the original function is maintained" means that a variant of gene or protein has a function (such as activity or property) corresponding to the function (such as activity or property) of the original gene or protein. The expression that "the original function is maintained" used for a gene means that a variant of the gene encodes a protein that maintains the original function. The expression that "the original function is maintained" used for a dicarboxylic acid efflux carrier gene may mean that a variant of the gene encodes a protein having a dicarboxylic acid-secreting activity. The expression that "the original function is maintained" used for a dicarboxylic acid efflux carrier may mean that the variant of the protein has a dicarboxylic acid-secreting activity.

[0066]

Examples of the dicarboxylic acid secreted by the dicarboxylic acid efflux carrier include the dicarboxylic acids mentioned above. The dicarboxylic acid efflux carrier may have a dicarboxylic acid-secreting activity for only a single kind of dicarboxylic acid, or may have a dicarboxylic acid-secreting activity for two or more kinds of dicarboxylic acids. In the present invention, combination of dicarboxylic acid efflux carrier and dicarboxylic acid secreted thereby is not particularly limited. The term "dicarboxylic acid-secreting activity" used for the *yeeA* gene and the YeeA protein may mean, for example, an activity for secreting a dicarboxylic acid selected from α -KG, malic acid, fumaric acid, succinic acid, and itaconic acid. The term "dicarboxylic acid-secreting activity" used for the *ynfM* gene and the YnfM protein may mean,

for example, an activity for secreting a dicarboxylic acid selected from α -KG, malic acid, fumaric acid, and succinic acid. The term "dicarboxylic acid-secreting activity" used for the *yjjP* gene, *yjjB* gene, YjjP protein, and YjjB protein may mean, for example, an activity for secreting succinic acid.

[0067]

It can be confirmed that a variant of a protein has a dicarboxylic acid-secreting activity by, for example, introducing a gene encoding the variant into a strain showing low succinic acid resistance such as the *P. ananatis* SC17(0) Δ sdhA strain, and confirming improvement of the succinic acid resistance. It can also be confirmed that a variant of a protein has a dicarboxylic acid-secreting activity by, for example, introducing a gene encoding the variant into a dicarboxylic acid-producing bacterium, and confirming improvement of dicarboxylic acid production amount.

[0068]

Hereafter, examples of the conservative variants will be explained.

[0069]

Homologues of a dicarboxylic acid efflux carrier gene or homologues of a dicarboxylic acid efflux carrier can be easily obtained from public databases by, for example, BLAST search or FASTA search using any of the nucleotide sequences of the dicarboxylic acid efflux carrier genes exemplified above or any of the amino acid sequences of dicarboxylic acid efflux carriers exemplified above as a query sequence. Further, homologues of a dicarboxylic acid efflux carrier gene can also be obtained by, for example, PCR using a chromosome of a microorganism such as bacteria as the template, and oligonucleotides prepared on

the basis of a nucleotide sequence of any one of those known dicarboxylic acid efflux carrier genes as primers.

[0070]

The dicarboxylic acid efflux carrier gene may be a gene (such as a DNA) encoding a protein having any of the aforementioned amino acid sequences (for example, the amino acid sequences of SEQ ID NOS: 2, 4, and 6 for the YeeA protein, the amino acid sequences of SEQ ID NOS: 8, 10, 12, 14, and 16 for the YnfM protein, the amino acid sequences of SEQ ID NOS: 18 and 20 for the YjjP protein, and the amino acid sequences of SEQ ID NOS: 22 and 24 for the YjjB protein) including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as the original function is maintained. For example, the encoded protein may have an extended or deleted N-terminus and/or C-terminus. Although the number meant by the term "one or several" used above may differ depending on the positions of amino acid residues in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it is, for example, 1 to 50, 1 to 40, or 1 to 30, preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3.

[0071]

The aforementioned substitution, deletion, insertion, or addition of one or several amino acid residues is a conservative mutation that maintains normal function of the protein. Typical examples of the conservative mutation are conservative substitutions. The conservative substitution is a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among

35

Leu, Ile, and Val, if the substitution site is a hydrophobic amino acid; between Gln and Asn, if the substitution site is a polar amino acid; among Lys, Arg, and His, if the substitution site is a basic amino acid; between Asp and Glu, if the substitution site is an acidic amino acid; and between Ser and Thr, if the substitution site is an amino acid having hydroxyl group. Examples of substitutions considered to be conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His, or Lys for Arg, substitution of Glu, Gln, Lys, His, or Asp for Asn, substitution of Asn, Glu, or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp, or Arg for Gln, substitution of Gly, Asn, Gln, Lys, or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg, or Tyr for His, substitution of Leu, Met, Val, or Phe for Ile, substitution of Ile, Met, Val, or Phe for Leu, substitution of Asn, Glu, Gln, His, or Arg for Lys, substitution of Ile, Leu, Val, or Phe for Met, substitution of Trp, Tyr, Met, Ile, or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe, or Trp for Tyr, and substitution of Met, Ile, or Leu for Val. Further, such substitution, deletion, insertion, addition, inversion, or the like of amino acid residues as mentioned above includes a naturally occurring mutation due to an individual difference, or a difference of species of the organism from which the gene is derived (mutant or variant).

[0072]

Further, the dicarboxylic acid efflux carrier gene may be a gene (such as a DNA) encoding a protein showing a homology

36

of, for example, 50% or more, 65% or more, or 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 99% or more, to the total amino acid sequence of any of the amino acid sequences mentioned above, so long as the original function is maintained. In this description, "homology" means "identity". [0073]

Further, the dicarboxylic acid efflux carrier gene may be a DNA that is able to hybridize under stringent conditions with a probe that can be prepared from any of the aforementioned nucleotide sequences (for example, the nucleotide sequences shown as SEQ ID NOS: 1, 3, and 5 for the *yeeA* gene, the nucleotide sequences shown as SEQ ID NOS: 7, 9, 11, 13, and 15 for the *ynfM* gene, the nucleotide sequences shown as SEQ ID NOS: 17 and 19 for the *yjjP* gene, and the nucleotide sequences shown as SEQ ID NOS: 21 and 23 for the *yjjB* gene), such as a sequence complementary to the whole sequence or a partial sequence of any of the aforementioned nucleotide sequences, so long as the original function is maintained. The "stringent conditions" refer to conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Examples of the stringent conditions include those under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 50%, 65%, or 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 97% homologous, particularly preferably not less than 99% homologous, hybridize to each other, and DNAs less homologous than the above do not hybridize to each other, or conditions of washing of typical Southern hybridization, i.e., conditions of washing once, .

37

preferably 2 or 3 times, at a salt concentration and temperature corresponding to 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably 0.1 x SSC, 0.1% SDS at 68°C.

[0074]

As described above, the probe used for the aforementioned hybridization may be a part of a sequence that is complementary to a gene. Such a probe can be prepared by PCR using oligonucleotides prepared on the basis of a known gene sequence as the primers and a DNA fragment containing any of the aforementioned genes as the template. For example, a DNA fragment having a length of about 300 bp can be used as the probe. When a DNA fragment having a length of about 300 bp is used as the probe, the washing conditions of the hybridization may be, for example, 50°C, 2 x SSC and 0.1% SDS.

[0075]

Further, since properties concerning degeneracy of codons changes depending on the host, the dicarboxylic acid efflux carrier gene may include substitution of corresponding codons for arbitrary codons. For example, the dicarboxylic acid efflux carrier gene may be modified to as to have codons optimum for the codon usage of a host to be used.

[0076]

The percentage of the sequence identity between two sequences can be determined by, for example, using a mathematical algorithm. Non-limiting examples of such a mathematical algorithm include the algorithm of Myers and Miller (1988) CABIOS 4:11-17, the local homology algorithm of Smith et al (1981) Adv. Appl. Math. 2:482, the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol.

48:443-453, the method for searching homology of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-2448, and an modified version of the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264, such as that described in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877.

[0077]

By using a program based on such a mathematical algorithm, sequence comparison (i.e. alignment) for determining the sequence identity can be performed. The program can be appropriately executed by a computer. Examples of such a program include, but not limited to, CLUSTAL of PC/Gene program (available from Intelligenetics, Mountain View, Calif.), ALIGN program (Version 2.0), and GAP, BESTFIT, BLAST, FASTA, and TFASTA of Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignment using these programs can be performed by using, for example, initial parameters. The CLUSTAL program is well described in Higgins et al. (1988) Gene 73:237-244 (1988), Higgins et al. (1989) CABIOS 5:151-153, Corpet et al. (1988) Nucleic Acids Res. 16:10881-90, Huang et al. (1992) CABIOS 8:155-65, and Pearson et al. (1994) Meth. Mol. Biol. 24:307-331.

[0078]

In order to obtain a nucleotide sequence homologous to a target nucleotide sequence, in particular, for example, BLAST nucleotide search can be performed by using BLASTN program with score of 100 and word length of 12. In order to obtain an amino acid sequence homologous to a target protein, in particular, for example, BLAST protein search can be performed by using

BLASTX program with score of 50 and word length of 3. See <http://www.ncbi.nlm.nih.gov> for BLAST nucleotide search and BLAST protein search. In addition, Gapped BLAST (BLAST 2.0) can be used in order to obtain an alignment including gap(s) for the purpose of comparison. In addition, PSI-BLAST can be used in order to perform repetitive search for detecting distant relationships between sequences. See Altschul et al. (1997) Nucleic Acids Res. 25:3389 for Gapped BLAST and PSI-BLAST. When using BLAST, Gapped BLAST, or PSI-BLAST, initial parameters of each program (e.g. BLASTN for nucleotide sequences, and BLASTX for amino acid sequences) can be used. Alignment can also be manually performed.

[0079]

The sequence identity between two sequences is calculated as a ratio of the same residues found at corresponding positions in the two sequences, when the two sequences are aligned so that the maximum coincidence of the residues is obtained for them.

[0080]

<1-3> Method for enhancing (increasing) expression of gene

The methods for enhancing (increasing) the expression of a gene such as dicarboxylic acid efflux carrier gene will be explained below.

[0081]

The expression "the expression of a gene is increased" means that the expression amount of the gene per cell is increased as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a reference strain that has not been modified so that the expression of an objective gene is increased. Examples of the non-modified strain include a wild-type strain and parent strain. The

expression "the expression of a gene is increased" may specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is increased, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is increased. The state that "the expression of a gene is increased" may also be referred to as "the expression of a gene is enhanced". The degree of the increase in the expression of a gene is not particularly limited, so long as the expression of the gene is increased as compared with that of a non-modified strain. The expression of a gene may be increased 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain. Further, the state that "the expression of a gene is increased" includes not only a state that the expression amount of an objective gene is increased in a strain that inherently expresses the objective gene, but also a state that the gene is introduced into a strain that does not inherently express the objective gene, and expressed therein. That is, the phrase "the expression of a gene is increased" may also mean, for example, that an objective gene is introduced into a strain that does not possess the gene, and is expressed therein. Further, so long as the expression of a gene is increased as a result, modification such as attenuation of the expression of an objective gene originally possessed by a host, or disruption of an objective gene originally possessed by a host may be performed, and then an appropriate type of the objective gene may be introduced into the host.

[0082]

The expression of a gene can be increased by, for example, increasing the copy number of the gene.

[0083]

The copy number of a gene can be increased by introducing the gene into the chromosome of a host. A gene can be introduced into a chromosome by, for example, using homologous recombination (Miller, J.H., Experiments in Molecular Genetics, 1972, Cold Spring Harbor Laboratory). Examples of the gene transfer method utilizing homologous recombination include, for example, a method using a linear DNA such as Red-driven integration (Datsenko, K.A., and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), a method of using a plasmid containing a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of using a suicide vector not having a replication origin that functions in a host, or a transduction method using a phage. Only one copy, or two or more copies of a gene may be introduced. For example, by performing homologous recombination using a sequence which is present in multiple copies on a chromosome as a target, multiple copies of a gene can be introduced into the chromosome. Examples of such a sequence which is present in multiple copies on a chromosome include repetitive DNAs, and inverted repeats located at the both ends of a transposon. Alternatively, homologous recombination may be performed by using an appropriate sequence on a chromosome such as a gene unnecessary for the production of an objective substance as a target. Further, a gene can also be randomly introduced into a chromosome by using a transposon or Mini-Mu (Japanese Patent Laid-open (Kokai) No. 2-109985, U.S. Patent No. 5,882,888, EP 805867 B1).

[0084]

Introduction of a target gene into a chromosome can be

confirmed by Southern hybridization using a probe having a sequence complementary to the whole gene or a part thereof, PCR using primers prepared on the basis of the sequence of the gene, or the like.

[0085]

Further, the copy number of a gene can also be increased by introducing a vector containing the gene into a host. For example, the copy number of a target gene can be increased by ligating a DNA fragment containing the target gene with a vector that functions in a host to construct an expression vector of the gene, and transforming the host with the expression vector. The DNA fragment containing the target gene can be obtained by, for example, PCR using the genomic DNA of a microorganism having the target gene as the template. As the vector, a vector autonomously replicable in the cell of the host can be used. The vector is preferably a multi-copy vector. Further, the vector preferably has a marker such as an antibiotic resistance gene for selection of transformant. Further, the vector may have a promoter and/or terminator for expressing the introduced gene. The vector may be, for example, a vector derived from a bacterial plasmid, a vector derived from a yeast plasmid, a vector derived from a bacteriophage, cosmid, phagemid, or the like. Specific examples of vector autonomously replicable in *Enterobacteriaceae* bacteria such as *Escherichia coli* include, for example, pUC19, pUC18, pHSG299, pHSG399, pHSG398, pBR322, pSTV29 (all of these are available from Takara Bio), pACYC184, pMW219 (NIPPON GENE), pTrc99A (Pharmacia), pPROK series vectors (Clontech), pKK233-2 (Clontech), pET series vectors (Novagen), pQE series vectors (QIAGEN), pACYC series vectors, and the broad host spectrum vector RSF1010. Specific examples of vector

autonomously replicable in coryneform bacteria include pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)); pAM330 (Agric. Biol. Chem., 48, 2901-2903 (1984)); plasmids obtained by improving these and having a drug resistance gene; plasmid pCRY30 described in Japanese Patent Laid-open (Kokai) No. 3-210184; plasmids pCRY21, pCRY2KE, pCRY2KX, pCRY31, pCRY3KE, and pCRY3KX described in Japanese Patent Laid-open (Kokai) No. 2-72876 and U.S. Patent No. 5,185,262; plasmids pCRY2 and pCRY3 described in Japanese Patent Laid-open (Kokai) No. 1-191686; pAJ655, pAJ611, and pAJ1844 described in Japanese Patent Laid-open (Kokai) No. 58-192900; pCG1 described in Japanese Patent Laid-open (Kokai) No. 57-134500; pCG2 described in Japanese Patent Laid-open (Kokai) No. 58-35197; pCG4 and pCG11 described in Japanese Patent Laid-open (Kokai) No. 57-183799; pVK7 described in Japanese Patent Laid-open (Kokai) No. 10-215883; and pVC7 described in Japanese Patent Laid-open (Kokai) No. 9-070291.

[0086]

When a gene is introduced, it is sufficient that the gene is expressibly harbored by the bacterium of the present invention. Specifically, it is sufficient that the gene is introduced so that it is expressed under control by a promoter sequence that functions in the bacterium of the present invention. The promoter may be a promoter derived from the host, or a heterogenous promoter. The promoter may be the native promoter of the gene to be introduced, or a promoter of another gene. As the promoter, for example, such a stronger promoter as mentioned later may also be used.

[0087]

A terminator for terminating the gene transcription can

be provided downstream of the gene. The terminator is not particularly limited so long as it functions in the bacterium of the present invention. The terminator may be a terminator derived from the host, or may be a heterogenous terminator. The terminator may be the native terminator of the gene to be introduced, or may be a terminator of another gene. Specific examples of the terminator include, for example, T7 terminator, T4 terminator, fd phage terminator, tet terminator, and trpA terminator.

[0088]

Vectors, promoters, and terminators available in various microorganisms are disclosed in detail in "Fundamental Microbiology Vol. 8, Genetic Engineering, KYORITSU SHUPPAN CO., LTD, 1987", and those can be used.

[0089]

Further, when two or more of genes are introduced, it is sufficient that the genes each are expressibly harbored by the bacterium of the present invention. For example, all the genes may be carried by a single expression vector or a chromosome. Further, the genes may be separately carried by two or more expression vectors, or separately carried by a single or two or more expression vectors and a chromosome. An operon constituted by two or more genes may also be introduced. The case of "introducing two or more genes" include, for example, cases of introducing two or more kinds of genes selected from the dicarboxylic acid efflux carrier genes, introducing genes coding for two or more kinds of proteins (such as enzymes), introducing genes coding for two or more subunits constituting a single protein complex (such as a single enzyme complex), and a combination of the foregoing cases.

[0090]

The gene to be introduced is not particularly limited so long as it codes for a protein that functions in the host. The gene to be introduced may be a gene derived from the host, or may be a heterogenous gene. When two or more kinds of genes are introduced, these genes may be derived from one kind of organism, or may be derived from two or more kinds of different organisms. That is, for example, when two or more kinds of genes selected from the dicarboxylic acid efflux carrier genes are introduced, all of the two or more kinds of genes may be derived from the same organism, or the two or more kinds of genes each may be derived from different organisms.

[0091]

The gene to be introduced can be obtained by, for example, PCR using primers designed on the basis of the nucleotide sequence of the gene, and using the genomic DNA of an organism having the gene, a plasmid carrying the gene, or the like as the template. The gene to be introduced may also be totally synthesized, for example, on the basis of the nucleotide sequence of the gene (Gene, 60(1), 115-127 (1987)). The obtained gene can be used as it is, or after being modified as required.

[0092]

Further, the expression of a gene can be increased by improving the transcription efficiency of the gene. In addition, the expression of a gene can also be increased by improving the translation efficiency of the gene. The transcription efficiency of the gene and the translation efficiency of the gene can be improved by, for example, modifying an expression control sequence of the gene. The term

"expression control sequence" collectively refers to sites that affect the expression of a gene. Examples of the expression control sequence include, for example, promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)), and spacer region between RBS and the start codon. Expression control sequences can be identified by using a promoter search vector or gene analysis software such as GENETYX. These expression control sequences can be modified by, for example, a method of using a temperature sensitive vector, or the Red driven integration method (WO2005/010175). [0093]

The transcription efficiency of a gene can be improved by, for example, replacing the promoter of the gene on a chromosome with a stronger promoter. The "stronger promoter" means a promoter providing an improved transcription of a gene compared with an inherently existing wild-type promoter of the gene. Examples of stronger promoters include, for example, the known high expression promoters such as T7 promoter, *trp* promoter, *lac* promoter, *thr* promoter, *tac* promoter, *trc* promoter, *tet* promoter, *araBAD* promoter, *rpoH* promoter, PR promoter, and PL promoter. Examples of stronger promoters usable in coryneform bacteria include the artificially modified P54-6 promoter (Appl. Microbiol. Biotechnolo., 53, 674-679 (2000)), *pta*, *aceA*, *aceB*, *adh*, and *amyE* promoters inducible in coryneform bacteria with acetic acid, ethanol, pyruvic acid, or the like, *cspB*, *SOD*, and *tuf* (EF-Tu) promoters, which are potent promoters capable of providing a large expression amount in coryneform bacteria (Journal of Biotechnology, 104 (2003) 311-323; Appl. Environ. Microbiol., 2005 Dec; 71 (12):8587-96), as well as *lac* promoter, *tac* promoter, and *trc* promoter.

Further, as the stronger promoter, a highly-active type of an existing promoter may also be obtained by using various reporter genes. For example, by making the -35 and -10 regions in a promoter region closer to the consensus sequence, the activity of the promoter can be enhanced (WO00/18935). Examples of highly active-type promoter include various tac-like promoters (Katashkina JI et al., Russian Federation Patent Application No. 2006134574) and *pnlp8* promoter (WO2010/027045). Methods for evaluating the strength of promoters and examples of strong promoters are described in the paper of Goldstein et al. (Prokaryotic Promoters in Biotechnology, Biotechnol. Annu. Rev., 1, 105-128 (1995)), and so forth.

[0094]

The translation efficiency of a gene can be improved by, for example, replacing the Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)) for the gene on a chromosome with a stronger SD sequence. The "stronger SD sequence" means a SD sequence that provides an improved translation of mRNA compared with the inherently existing wild-type SD sequence of the gene. Examples of stronger SD sequences include, for example, RBS of the gene 10 derived from phage T7 (Olins P.O. et al, Gene, 1988, 73, 227-235). Further, it is known that substitution, insertion, or deletion of several nucleotides in a spacer region between RBS and the start codon, especially in a sequence immediately upstream of the start codon (5'-UTR), significantly affects the stability and translation efficiency of mRNA, and hence, the translation efficiency of a gene can also be improved by modifying them.

[0095]

The translation efficiency of a gene can also be improved

by, for example, modifying codons. In *Escherichia coli* etc., a clear codon bias exists among the 61 amino acid codons found within the population of mRNA molecules, and the level of cognate tRNA appears directly proportional to the frequency of codon usage (Kane, J.F., Curr. Opin. Biotechnol., 6 (5), 494-500 (1995)). That is, if there is a large amount of mRNA containing an excess amount of rare codons, a translational problem may arise. According to the recent researches, it is suggested that clusters of AGG/AGA, CUA, AUA, CGA, or CCC codons may especially reduce both the quantity and quality of a synthesized protein. Such a problem occurs especially at the time of expression of a heterologous gene. Therefore, in the case of heterogenous expression of a gene or the like, the translation efficiency of the gene can be improved by replacing a rare codon present in the gene with a synonymous codon more frequently used. That is, the gene to be introduced may be modified, for example, so as to contain optimal codons according to the frequencies of codons observed in a host to be used. Codons can be replaced by, for example, the site-specific mutation method for introducing an objective mutation into an objective site of DNA. Examples of the site-specific mutation method include the method utilizing PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter, P., Meth. in Enzymol., 154, 382 (1987)), and the method utilizing phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)). Alternatively, a gene fragment in which objective codons are replaced may be totally synthesized. Frequencies of codons in various organisms are disclosed in the "Codon Usage Database" (<http://www.kazusa.or.jp/codon>; Nakamura, Y. et al, Nucl.

Acids Res., 28, 292 (2000)).

[0096]

Further, the expression of a gene can also be increased by amplifying a regulator that increases the expression of the gene, or deleting or attenuating a regulator that reduces the expression of the gene.

[0097]

Such methods for increasing the gene expression as mentioned above may be used independently or in an arbitrary combination.

[0098]

The method for the transformation is not particularly limited, and conventionally known methods can be used. There can be used, for example, a method of treating recipient cells with calcium chloride so as to increase the permeability thereof for DNA, which has been reported for the *Escherichia coli* K-12 strain (Mandel, M. and Higa, A., J. Mol. Biol., 1970, 53, 159-162), and a method of preparing competent cells from cells which are in the growth phase, followed by transformation with DNA, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1977, 1:153-167).

Alternatively, there can also be used a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing a recombinant DNA into the DNA-recipient cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes, and yeasts (Chang, S. and Choen, S.N., 1979, Mol. Gen. Genet., 168:111-115; Bibb, M.J., Ward, J.M. and Hopwood, O.A., 1978, Nature, 274:398-400; Hinnen, A., Hicks, J.B. and Fink, G.R., 1978, Proc. Natl. Acad. Sci. USA, 75:1929-1933). Further, the

electric pulse method reported for coryneform bacteria (Japanese Patent Laid-open (Kokai) No. 2-207791) can also be used.

[0099]

An increase in the expression of a gene can be confirmed by confirming an increase in the transcription amount of the gene, or by confirming an increase in the amount of a protein expressed from the gene. An increase in the expression of a gene can also be confirmed by confirming an increase in the activity of the protein expressed from the gene.

[0100]

An increase of the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain such as a wild-type strain or parent strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Sambrook, J., et al., Molecular Cloning A Laboratory Manual/Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA may increase, for example, 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain.

[0101]

An increase in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of the protein may increase, for example, 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain.

[0102]

An increase in the activity of a protein can be confirmed

by measuring the activity of the protein. The activity of a protein may be increased, for example, 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain.

[0103]

The aforementioned methods for increasing the expression of a gene can be used for enhancement of the expression of arbitrary genes such as a gene encoding an enzyme of the dicarboxylic acid biosynthesis system, in addition to the enhancement of the expression of a dicarboxylic acid efflux carrier gene.

[0104]

<1-4> Methods for increasing activity of protein

The methods for increasing the activity of a protein will be explained below.

[0105]

The expression "the activity of a protein is increased" means that the activity of the protein per cell is increased as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a reference strain that has not been modified so that the activity of an objective protein is increased. Examples of the non-modified strain include a wild-type strain and parent strain. The state that "the activity of a protein is increased" may also be expressed as "the activity of a protein is enhanced". Specifically, the expression "the activity of a protein is increased" means that the number of molecules of the protein per cell is increased, and/or the function of each molecule of the protein is increased as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein

is increased" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) coding for the protein, or the translation amount of the protein (i.e. the amount of the protein). Further, the state that "the activity of a protein is increased" includes not only a state that the activity of an objective protein is increased in a strain inherently having the activity of the objective protein, but also a state that the activity of an objective protein is imparted to a strain not inherently having the activity of the objective protein. Further, so long as the activity of the protein is eventually increased, the activity of an objective protein inherently contained in a host may be attenuated and/or eliminated, and then an appropriate type of the objective protein may be imparted to the host.

[0106]

The degree of the increase in the activity of a protein is not particularly limited, so long as the activity of the protein is increased as compared with a non-modified strain. The activity of the protein may be increased 1.5 times or more, 2 times or more, or 3 times or more, as compared with that of a non-modified strain. Further, when the strain before the modification does not have the activity of the objective protein, it is sufficient that the protein is produced as a result of introduction of the gene encoding the protein, and for example, the protein may be produced to such an extent that the enzyme activity can be measured.

[0107]

The modification for increasing the activity of a protein is attained by, for example, increasing the expression of a gene

coding for the protein. The method for increasing expression of a gene is as described above.

[0108]

Further, the modification that increases the activity of a protein can also be attained by, for example, enhancing the specific activity of the enzyme. Enhancement of the specific activity also includes desensitization to feedback inhibition. That is, when a protein is subject to feedback inhibition by a metabolite, the activity of the protein can be increased by making the bacterium harbor a gene encoding a mutant protein that has been desensitized to the feedback inhibition. In the present invention, "desensitization to feedback inhibition" includes complete elimination of the feedback inhibition, and attenuation of the feedback inhibition, unless otherwise stated. A protein showing an enhanced specific activity can be obtained by, for example, searching various organisms. Further, a highly-active type of an existing protein may also be obtained by introducing a mutation into the existing protein. The mutation to be introduced may be, for example, substitution, deletion, insertion, or addition of one or several amino acid residues at one or several position of the protein. The mutation can be introduced by, for example, such a site-specific mutation method as mentioned above. The mutation may also be introduced by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include irradiation of X-ray, irradiation of ultraviolet, and a treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS). Further, a random mutation may be induced by directly treating DNA in vitro with hydroxylamine. Enhancement of the

specific activity may be independently used, or may be used in an arbitrary combination with such methods for enhancing gene expression as mentioned above.

[0109]

In addition, when a protein functions as a complex consisting of a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually increased. That is, for example, when the activity of a protein is increased by increasing the expression of a gene, the expression of a part or all of the plurality of genes that code for the subunits may be enhanced. It is usually preferable to enhance the expression of all of the plurality of genes coding for the subunits. Further, the subunits constituting the complex may be derived from a single kind of organism or two or more kinds of organisms, so long as the complex has a function of the objective protein. That is, for example, genes of the same organism coding for a plurality of subunits may be introduced into a host, or genes of different organisms coding for a plurality of subunits may be introduced into a host.

[0110]

An increase in the activity of a protein can be confirmed by measuring the activity of the protein. An increase in the activity of a protein can also be confirmed by confirming an increase in the expression of a gene coding for the protein.

[0111]

The aforementioned methods for increasing the activity of a protein can be used for enhancement of activities of arbitrary proteins such as dicarboxylic acid biosynthesis system enzymes, and enhancement of the expression of arbitrary

genes such as genes encoding those arbitrary proteins.

[0112]

<1-5> Method for reducing activity of protein

The methods for reducing activity of a protein will be explained below.

[0113]

The expression "the activity of a protein is reduced" means that the activity of the protein per cell is reduced as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a reference strain that has not been modified so that the activity of an objective protein is reduced. Examples of the non-modified strain include a wild-type strain or parent strain. The state that "the activity of a protein is reduced" also includes a state that the activity of the protein has completely disappeared. Specifically, the expression "the activity of a protein is reduced" means that the number of molecules of the protein per cell is reduced, and/or the function of each molecule of the protein is reduced as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is reduced" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) coding for the protein or the translation amount of the protein (i.e. the amount of the protein). The state that "the number of molecules of the protein per cell is reduced" also includes a state that the protein does not exist at all. The state that "the function of each molecule of the protein is reduced" also includes a state that the function of each protein molecule has completely disappeared. The degree of the reduction in the activity of

a protein is not particularly limited, so long as the activity is reduced as compared with that of a non-modified strain. The activity of a protein may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0114]

The modification for reducing the activity of a protein can be attained by, for example, reducing the expression of a gene coding for the protein. The expression "the expression of a gene is reduced" means that the expression of the gene per cell is reduced as compared with that of a non-modified strain such as a wild-type strain and parent strain. The expression "the expression of a gene is reduced" may specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is reduced, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is reduced. The state that "the expression of a gene is reduced" also includes a state that the gene is not expressed at all. The state that "the expression of a gene is reduced" is also referred to as "the expression of a gene is attenuated". The degree of the reduction in the expression of a gene is not particularly limited, so long as the expression is reduced as compared with that of a non-modified strain. The expression of a gene may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0115]

The reduction in gene expression may be due to, for example, a reduction in the transcription efficiency, a reduction in the translation efficiency, or a combination of them. The expression of a gene can be reduced by modifying an expression

control sequence of the gene such as promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome-binding site (RBS)), and spacer region between RBS and the start codon of the gene. When an expression control sequence is modified, preferably one or more nucleotides, more preferably two or more nucleotides, particularly preferably three or more nucleotides, of the expression control sequence are modified. Further, a part or the whole of an expression control sequence may be deleted. The expression of a gene can also be reduced by, for example, manipulating a factor responsible for expression control. Examples of the factor responsible for expression control include low molecules responsible for transcription or translation control (inducers, inhibitors, etc.), proteins responsible for transcription or translation control (transcription factors etc.), nucleic acids responsible for transcription or translation control (siRNA etc.), and so forth. Further, the expression of a gene can also be reduced by, for example, introducing a mutation that reduces the expression of the gene into the coding region of the gene. For example, the expression of a gene can be reduced by replacing a codon in the coding region of the gene with a synonymous codon used less frequently in a host. Further, for example, the gene expression may be reduced due to disruption of a gene as described later.

[0116]

The modification for reducing the activity of a protein can also be attained by, for example, disrupting a gene coding for the protein. The expression "a gene is disrupted" means that a gene is modified so that a protein that can normally function is not produced. The state that "a protein that

normally functions is not produced" includes a state that the protein is not produced at all from the gene, and a state that the protein of which the function (such as activity or property) per molecule is reduced or eliminated is produced from the gene. [0117]

Disruption of a gene can be attained by, for example, deleting a part or the whole of the coding region of the gene on a chromosome. Furthermore, the whole of a gene including sequences upstream and downstream from the gene on a chromosome may be deleted. The region to be deleted may be any region such as an N-terminus region, an internal region, or a C-terminus region, so long as the activity of the protein can be reduced. Deletion of a longer region can usually more surely inactivate the gene. Further, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same.

[0118]

Disruption of a gene can also be attained by, for example, introducing a mutation for an amino acid substitution (missense mutation), a stop codon (nonsense mutation), a frame shift mutation which adds or deletes one or two nucleotide residues, or the like into the coding region of the gene on a chromosome (Journal of Biological Chemistry, 272:8611-8617 (1997); Proceedings of the National Academy of Sciences, USA, 95 5511-5515 (1998); Journal of Biological Chemistry, 26 116, 20833-20839 (1991)).

[0119]

Disruption of a gene can also be attained by, for example, inserting another sequence into a coding region of the gene on a chromosome. Site of the insertion may be in any region of

the gene, and insertion of a longer region can usually more surely inactivate the gene. It is preferred that reading frames of the sequences upstream and downstream from the insertion site are not the same. The other sequence is not particularly limited so long as a sequence that reduces or eliminates the activity of the encoded protein is chosen, and examples thereof include, for example, a marker gene such as antibiotic resistance genes, and a gene useful for production of an objective substance.

[0120]

Such modification of a gene on a chromosome as described above can be attained by, for example, preparing a deficient type gene modified so that it is unable to produce a protein that normally functions, and transforming a host with a recombinant DNA containing the deficient type gene to cause homologous recombination between the deficient type gene and the wild-type gene on a chromosome and thereby substitute the deficient type gene for the wild-type gene on the chromosome. In this procedure, if a marker gene selected according to the characteristics of the host such as auxotrophy is included in the recombinant DNA, the operation becomes easier. Examples of the deficient type gene include a gene including deletion of all or a part of the gene, gene including a missense mutation, gene including a nonsense mutation, gene including a frame shift mutation, and gene including insertion of a transposon or marker gene. The protein encoded by the deficient type gene has a conformation different from that of the wild-type protein, even if it is produced, and thus the function thereof is reduced or eliminated. Such gene disruption based on gene substitution utilizing homologous recombination has already been

established, and there are methods of using a linear DNA such as a method called "Red driven integration" (Datsenko, K.A, and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), and a method utilizing the Red driven integration in combination with an excision system derived from λ phage (Cho, E.H., Gumport, R.I., Gardner, J.F., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175), a method of using a plasmid having a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of utilizing a suicide vector not having a replication origin that functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

[0121]

Modification for reducing activity of a protein can also be attained by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include irradiation of X-ray or ultraviolet and treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS).

[0122]

When a protein functions as a complex consisting of a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that code for the respective subunits may be disrupted or the like. Further, when there is a plurality of isozymes of a protein, a part or all of the activities of the plurality of isozymes may be reduced, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that code for the respective

isozymes may be disrupted or the like.

[0123]

A reduction in the activity of a protein can be confirmed by measuring the activity of the protein.

[0124]

A reduction in the activity of a protein can also be confirmed by confirming a reduction in the expression of a gene coding for the protein. A reduction in the expression of a gene can be confirmed by confirming a reduction in the transcription amount of the gene or a reduction in the amount of the protein expressed from the gene.

[0125]

A reduction in the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that of a non-modified strain.

[0126]

A reduction in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA) 2001). The amount of the protein is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that of a non-modified strain.

[0127]

Disruption of a gene can be confirmed by determining

nucleotide sequence of a part or the whole of the gene, restriction enzyme map, full length, or the like of the gene depending on the means used for the disruption.

[0128]

The aforementioned methods for reducing the activity of a protein as mentioned above can also be applied to reduction in the activities of arbitrary proteins such as an enzyme that catalyzes a reaction branching off from the biosynthesis pathway of an objective dicarboxylic acid to generate a compound other than the objective dicarboxylic acid, and reduction in the expression of arbitrary genes such as genes encoding those arbitrary proteins.

[0129]

<2> Method for producing dicarboxylic acid of the present invention

The method of the present inventions is a method for producing a dicarboxylic acid comprising culturing the bacterium of the present invention in a medium to produce and accumulate the dicarboxylic acid in the medium, and collecting the dicarboxylic acid from the medium. In the present invention, one kind of dicarboxylic acid may be produced, or two or more kinds of dicarboxylic acids may be produced.

[0130]

The medium to be used is not particularly limited, so long as the bacterium of the present invention can proliferate in the medium and produce a dicarboxylic acid. As the medium, for example, a usual medium used for culture of bacteria such as those belonging to the family *Enterobacteriaceae* and coryneform bacteria can be used. The medium may contain carbon source, nitrogen source, phosphorus source, and sulfur source, as well

as other components selected from various organic components and inorganic components as required. The types and concentrations of the medium components can be appropriately determined according to various conditions such as the type of the bacterium to be used and the type of the dicarboxylic acid to be produced.

[0131]

Specific examples of the carbon source include, for example, saccharides such as glucose, fructose, sucrose, lactose, galactose, xylose, arabinose, blackstrap molasses, hydrolysate of starches, and hydrolysate of biomass, organic acids such as acetic acid, and citric acid, alcohols such as ethanol, glycerol, and crude glycerol, and fatty acids. As the carbon source, plant-derived materials can be preferably used. Examples of the plant include, for example, corn, rice, wheat, soybean, sugarcane, beet, and cotton. Examples of the plant-derived materials include, for example, organs such as root, stem, trunk, branch, leaf, flower, and seed, plant bodies including them, and decomposition products of these plant organs. The forms of the plant-derived materials at the time of use thereof are not particularly limited, and they can be used in any form such as unprocessed product, juice, ground product, and purified product. Pentoses such as xylose, hexoses such as glucose, or mixtures of them can be obtained from, for example, plant biomass, and used. Specifically, these saccharides can be obtained by subjecting a plant biomass to such a treatment as steam treatment, hydrolysis with concentrated acid, hydrolysis with diluted acid, hydrolysis with an enzyme such as cellulase, and alkaline treatment. Since hemicellulose is generally more easily hydrolyzed

compared with cellulose, hemicellulose in a plant biomass may be hydrolyzed beforehand to liberate pentoses, and then cellulose may be hydrolyzed to generate hexoses. Further, xylose may be supplied by conversion from hexoses by, for example, imparting a pathway for converting hexose such as glucose to xylose to the bacterium of the present invention. As the carbon source, one kind of carbon source may be used, or two or more kinds of carbon sources may be used in combination.

[0132]

The concentration of the carbon source in the medium is not particularly limited, so long as the bacterium of the present invention can proliferate and produce a dicarboxylic acid. It is preferable to make the concentration of the carbon source in the medium as high as possible within such a range that production of the dicarboxylic acid is not inhibited. Initial concentration of the carbon source in the medium may be, for example, usually 1 to 30% (w/v), preferably 3 to 10% (w/v). Further, in accordance with consumption of the carbon source accompanying progress of the fermentation, the carbon source may be additionally added.

[0133]

Specific examples of the nitrogen source include, for example, ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate, organic nitrogen sources such as peptone, yeast extract, meat extract, and soybean protein decomposition product, ammonia, and urea. Ammonia gas and aqueous ammonia used for pH adjustment may also be used as a nitrogen source. As the nitrogen source, one kind of nitrogen source may be used, or two or more kinds of nitrogen sources

may be used in combination.

[0134]

Specific examples of the phosphate source include, for example, phosphate salts such as potassium dihydrogenphosphate and dipotassium hydrogenphosphate, and phosphoric acid polymers such as pyrophosphoric acid. As the phosphate source, one kind of phosphate source may be used, or two or more kinds of phosphate sources may be used in combination.

[0135]

Specific examples of the sulfur source include, for example, inorganic sulfur compounds such as sulfates, thiosulfates, and sulfites, and sulfur-containing amino acids such as cysteine, cystine, and glutathione. As the sulfur source, one kind of sulfur source may be used, or two or more kinds of sulfur sources may be used in combination.

[0136]

Specific examples of other various organic and inorganic components include, for example, inorganic salts such as sodium chloride and potassium chloride; trace metals such as iron, manganese, magnesium and calcium; vitamins such as vitamin B1, vitamin B2, vitamin B6, nicotinic acid, nicotinamide, and vitamin B12; amino acids; nucleic acids; and organic components containing these such as peptone, casamino acid, yeast extract, and soybean protein decomposition product. As the other various organic and inorganic components, one kind of component may be used, or two or more kinds of components may be used in combination.

[0137]

Further, when an auxotrophic mutant strain that requires an amino acid or the like for growth thereof is used, it is

preferable to supplement a required nutrient to the medium. For example, an itaconic acid-producing bacterium may require L-glutamic acid due to deficiency of isocitrate dehydrogenase etc. In such a case, it is preferable to supplementarily add L-glutamic acid to the medium.

[0138]

Culture conditions are not particularly limited, so long as the bacterium of the present invention can proliferate and produce a dicarboxylic acid. The culture can be performed with, for example, usual conditions used for culture of bacteria such as those belonging to the family *Enterobacteriaceae* or coryneform bacteria. The culture conditions may be appropriately determined according to various conditions such as the type of the bacterium to be used and the type of the dicarboxylic acid to be produced.

[0139]

The culture can be performed by using a liquid medium. At the time of the culture, the bacterium of the present invention cultured on a solid medium such as agar medium may be directly inoculated into a liquid medium, or the bacterium of the present invention cultured in a liquid medium as seed culture may be inoculated into a liquid medium for main culture. That is, the culture may be performed separately as seed culture and main culture. In such a case, the culture conditions of the seed culture and the main culture may be or may not be the same. The amount of the bacterium of the present invention contained in the medium at the time of the start of the culture is not particularly limited. For example, seed culture showing an OD₆₆₀ of 4 to 8 may be added to a medium for main culture at a ratio of 0.1 to 30 mass %, preferably 1 to 10 mass %, at

the time of the start of the culture.

[0140]

The culture can be performed as batch culture, fed-batch culture, continuous culture, or a combination of these. The medium used at the start of the culture is also referred to as "starting medium". The medium supplied to the culture system (fermentation tank) in the fed-batch culture or the continuous culture is also referred to as "feed medium". To supply a feed medium to the culture system in the fed-batch culture or the continuous culture is also referred to as "feed". Further, when the culture is performed separately as seed culture and main culture, the culture schemes of the seed culture and the main culture may be or may not be the same. For example, both the seed culture and the main culture may be performed as batch culture. Alternatively, for example, the seed culture may be performed as batch culture, and the main culture may be performed as fed-batch culture or continuous culture.

[0141]

The culture may be performed under an aerobic condition, microaerobic condition, or anaerobic condition. The culture is preferably performed under a microaerobic condition or anaerobic condition. The aerobic condition means that dissolved oxygen concentration in the liquid medium is not lower than 0.33 ppm, which is the detection limit for the detection with an oxygen membrane electrode, preferably not lower than 1.5 ppm. The microaerobic condition means that, although oxygen is supplied to the culture system, dissolved oxygen concentration in the liquid medium is lower than 0.33 ppm. The anaerobic condition means a condition that oxygen is not supplied to the culture system. The culture may be performed

under the condition chosen above during the whole culture period, or during only a part of the culture period. That is, "to culture under an aerobic condition" means that the culture is performed under an aerobic condition during at least a part of the whole culture period. Further, "to culture under a microaerobic condition" means that the culture is performed under a microaerobic condition during at least a part of the whole culture period. Furthermore, "to culture under an anaerobic condition" means that the culture is performed under an anaerobic condition during at least a part of the whole culture period. The "part of the whole culture period" may be, for example, a period of 50% or more, 70 or more, 80% or more, 90% or more, 95% or more, or 99% or more, of the whole culture period. When the culture is performed separately as seed culture and main culture, the "whole culture period" may mean the whole period of the main culture. Specifically, the culture under an aerobic condition can be performed by aeration culture or shaking culture. The microaerobic condition or anaerobic condition can be attained by means of reducing aeration volume or stirring velocity, performing the culture in a sealed vessel without aeration, aerating an inert gas containing carbon dioxide gas, or the like to reduce dissolved oxygen concentration in the liquid medium.

[0142]

The pH of the medium may be, for example, 3 to 10, preferably 4.0 to 9.5. The pH of the medium can be adjusted during the culture as required. The pH of the medium can be adjusted by using various alkaline and acidic substances such as ammonia gas, aqueous ammonia, sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate,

magnesium carbonate, sodium hydroxide, calcium hydroxide, and magnesium hydroxide.

[0143]

The medium may contain carbonate ions, bicarbonate ions, carbon dioxide gas, or a combination of these. These components may be supplied, for example, by metabolism of the bacterium of the present invention, or from carbonate salt and/or bicarbonate salt used for pH adjustment. These components may also be supplied by further adding carbonic acid, bicarbonic acid, salts thereof, or carbon dioxide gas, as required. Specific examples of salts of carbonic acid or bicarbonic acid include, for example, calcium carbonate, magnesium carbonate, ammonium carbonate, sodium carbonate, potassium carbonate, ammonium bicarbonate, sodium bicarbonate, and potassium bicarbonate. Carbonate ions and/or bicarbonate ions may be added at a concentration of 0.001 to 5 M, preferably 0.1 to 3 M, more preferably 1 to 2 M. When carbon dioxide gas is contained, carbon dioxide gas may be contained in an amount of 50 mg to 25 g, preferably 100 mg to 15 g, more preferably 150 mg to 10 g, per liter of the solution.

[0144]

The culture temperature may be, for example, 20 to 45°C, preferably 25 to 37°C. The culture time may be, for example, 10 to 120 hours. The culture may be continued, for example, until the carbon source contained in the medium is consumed, or until the activity of the bacterium of the present invention is lost.

[0145]

By culturing the bacterium of the present invention under such conditions as described above, a dicarboxylic acid is

accumulated in the medium.

[0146]

Production of a dicarboxylic acid can be confirmed by known methods used for detection or identification of compounds. Examples of such methods include, for example, HPLC, LC/MS, GC/MS, and NMR. These methods can be independently used, or can be used in an appropriate combination.

[0147]

The produced dicarboxylic acid can be collected by known methods used for separation and purification of compounds. Examples of such methods include, for example, ion-exchange resin method, membrane treatment, precipitation, and crystallization. These methods can be independently used, or can be used in an appropriate combination. When a dicarboxylic acid is accumulated in cells of the bacterium, the cells can be disrupted with, for example, ultrasonic waves or the like, and then the dicarboxylic acid can be collected by the ion exchange resin method or the like from supernatant obtained by removing the cells from the cell-disrupted suspension by centrifugation. The collected dicarboxylic acid may be a free compound, a salt thereof, or a mixture of them. That is, the term "dicarboxylic acid" used in the present invention means a free dicarboxylic acid, a salt thereof, or a mixture of them, unless otherwise stated. Examples of the salt include, for example, ammonium salt, sodium salt, and potassium salt.

[0148]

Further, when the dicarboxylic acid deposits in the medium, it can be collected by centrifugation or filtration.

Dicarboxylic acid deposited in the medium and dicarboxylic acid dissolving in the medium may be isolated together after the

dicarboxylic acid dissolving in the medium is crystallized.
[0149]

The collected dicarboxylic acid may contain, for example, bacterial cells, medium components, moisture, and by-product metabolites of the bacterium, in addition to the dicarboxylic acid. Purity of the collected dicarboxylic acid may be, for example, 30% (w/w) or higher, 50% (w/w) or higher, 70% (w/w) or higher, 80% (w/w) or higher, 90% (w/w) or higher, or 95% (w/w) or higher.

Examples

[0150]

Hereafter, the present invention will be more specifically explained with reference to examples. However, the present invention is not limited by these examples.

[0151]

<Reference Example> Methods for genetic manipulation in *P. ananatis*

<1> Construction of *P. ananatis* SC17(0)/RSFRedTER strain

The *P. ananatis* SC17(0) strain (VKPM B-9246) was cultured overnight in the LB liquid medium. The SC17(0) strain was deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on September 21, 2005, and assigned an accession number VKPM B-9246. The culture broth (100 μ L) was inoculated into fresh LB liquid medium (4 mL), and shaking culture was performed at 34°C for 3 hours. The cells were collected, washed 3 times with 10% glycerol, and used as competent cells. A helper plasmid RSFRedTER (WO2008/090770A1) was introduced into the competent cells by electroporation.

RSFRedTER is also referred to as RSF-Red-TER. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . After the electroporation, the cells were cultured for 2 hours in the SOC medium (20 g/L of Bacto tryptone, 5 g/L of yeast extract, 0.5 g/L of NaCl, 10 g/L of glucose), applied to the LB agar medium containing 25 mg/L of chloramphenicol (Cm), and cultured at 34°C for 16 hours. As a result, a transformant that showed chloramphenicol resistance was obtained, and designated as SC17(0)/RSFRedTER strain.

[0152]

<2> Construction of gene-disrupted strain of *P. ananatis* SC17(0) strain

The SC17(0)/RSFRedTER strain was cultured overnight in the LB liquid medium containing 25 mg/L of chloramphenicol. The culture broth (1 mL) was inoculated into the LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. By PCR using a plasmid having a drug resistance gene (drug resistance gene cassette) flanked by *attL* and *attR* sequences of λ phage as the template, a DNA fragment for disrupting objective gene having sequences of 50 bp complementary to internal sequences of the objective gene at both ends, and the drug resistance gene cassette between them was amplified. This DNA fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the

conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . After the electroporation, the SOC medium cooled on ice was immediately added to the cells, restoration culture was performed at 34°C for 2 hours with shaking, and a strain in which the objective gene was replaced with the drug resistance gene cassette was selected on the LB agar medium containing the drug corresponding to the drug resistance gene.

[0153]

<3> Removal of RSFRedTER plasmid

If the RSFRedTER plasmid should be removed from a strain harboring this plasmid, the strain was streaked and cultured on a medium not containing the drug, and a colony that appeared was applied to the LB agar medium containing the M9 components (17.1 g/L of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 3 g/L of KH_2PO_4 , 0.5 g/L of NaCl, and 1 g/L of NH_4Cl), 10% sucrose, and 1 mM IPTG to obtain the strain from which the RSFRedTER plasmid was removed.

[0154]

<4> Removal of drug resistance gene

If the drug resistance gene should be removed from a strain containing this gene, the pMW-intxis-sacB(Cm) plasmid (see Reference Example <5>) was introduced into the strain by electroporation, and a transformant was selected on the LB agar medium containing 25 mg/L of chloramphenicol. The obtained transformant was streaked and cultured on an agar medium that did not contain any drug, replicated on a medium containing the drug corresponding to the drug resistance gene and a medium not containing the drug, and a strain in which the drug resistance gene was removed was selected.

[0155]

<5> Construction of pMW-intxis-sacB(Cm)

The pMW-intxis-sacB(Cm) plasmid for removal of drug resistance gene was constructed by inserting the chloramphenicol resistance gene and the *sacB* gene derived from RSFRedTER (WO2008/090770A1) into pMW-intxis-ts (WO2007/037460) at the *Pst*I-*Sph*I site. Specifically, a fragment of about 4.0 kb containing the chloramphenicol resistance gene and the *sacB* gene was amplified by PCR using RSFRedTER as the template and the primers of SEQ ID NOS: 25 and 26, and purified. Separately, pMW-intxis-ts was treated with *Pst*I and *Sph*I, blunt-ended, and phosphorylated by using BKL Kit (Takara). These two fragments were ligated by a ligation reaction, and used to transform the *E. coli* DH5 α strain, and a transformant was selected on an L agarose plate (10 g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5 g/L of NaCl, 2% agarose) containing 25 mg/L of chloramphenicol and 100 mg/L of ampicillin to obtain DH5 α /pMW-intxis-sacB(Cm) strain. From this strain, the plasmid pMW-intxis-sacB(Cm) was obtained in a conventional manner.

[0156]

<Example 1> Evaluation of effect of *yeeA* gene amplification in *P. ananatis* succinic acid-producing strain and α -KG-producing strain

In this example, the *yeeA* gene was obtained by screening for dicarboxylic acid efflux carrier gene, and effect of *yeeA* gene amplification on dicarboxylic acid production was evaluated by using succinic acid-producing strain derived from the *P. ananatis* SC17(0) strain and α -KG-producing strain derived from the *P. ananatis* SC17 strain as the hosts.

[0157]

75

<1-1> Screening for dicarboxylic acid efflux carrier gene

<1-1-1> Construction of *P. ananatis* SC17(0) Δ *sdhA* strain

First, a strain in which the *sdhA* gene encoding succinate dehydrogenase was disrupted was constructed by using the SC17(0) strain as the parent strain by the following method. PCR was performed by using the primers shown as SEQ ID NOS: 27 and 28, and pMW118-attL-Km^r-attR (WO2008/090770A1) as the template to amplify a DNA fragment for disruption of the *sdhA* gene containing the kanamycin (Km) resistance gene. By introducing this DNA fragment into the competent cells of the SC17(0)/RSFRedTER strain, and selecting a transformant on the LB agar medium containing 40 mg/L of kanamycin and 20 mM disodium malate, the SC17(0) Δ *sdhA*::Km strain was obtained, in which the *sdhA* gene was replaced with the Km resistance gene. The Km resistance gene was removed from this strain to obtain the SC17(0) Δ *sdhA* strain deficient in the *sdhA* gene.

[0158]

<1-1-2> Screening for dicarboxylic acid efflux carrier gene

The genomic library of the *P. ananatis* AJ13355 strain (FERM BP-6614) was introduced into the SC17(0) Δ *sdhA* strain, and screening for dicarboxylic acid efflux carrier gene was performed by using the succinic acid resistance as the marker. The AJ13355 strain was deposited at the Agency of Industrial Science and Technology, National Institute of Bioscience and Human-Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 19, 1998, and assigned an accession number of FERM P-16644. Then, the deposit was converted to an international

76

deposit under the provisions of Budapest Treaty on January 11, 1999, and assigned an accession number of FERM BP-6614. As a result, improvement in the succinic acid resistance was observed for the strain into which a plasmid containing the *yeeA* gene was introduced, and thus it was estimated that the *yeeA* gene was a dicarboxylic acid efflux carrier gene.

[0159]

<1-2> Evaluation of effect of *yeeA* gene amplification in *P. ananatis* succinic acid-producing strain

<1-2-1> Construction of succinic acid-producing bacterium, *P. ananatis* SC17(0) Δ *sdhA*/RSFPP strain

The RSFPP plasmid was obtained by removing the region containing the *gdhA* gene from the RSFPPG plasmid (WO2010/027022A1). Specifically, the RSFPPG plasmid was treated with the restriction enzyme *NspV*, subjected to a heat treatment at 75°C for 10 minutes for inactivation of the enzyme, and then self-ligated by using DNA Ligation Kit (Takara Bio). The *E. coli* DH5 α strain was transformed by using this DNA solution, and selection was performed on the LB agar medium containing 12.5 mg/L of tetracycline to obtain DH5 α /RSFPP strain. The plasmid RSFPP was obtained from this strain in a conventional manner. The RSFPP plasmid is an expression plasmid for the *prpC* gene encoding methyl citrate synthase and the *ppc* gene encoding phosphoenolpyruvate carboxylase. The RSFPP plasmid can be used for enhancing carbon flow into the TCA cycle.

[0160]

By introducing the RSFPP plasmid into the SC17(0) Δ *sdhA* strain, and selecting transformants on the LBGM9 agar medium (10 g/L of Bacto tryptone, 5 g/L of yeast extract, 10 g/L of

NaCl, 6 g/L of Na₂HPO₄, 3 g/L of KH₂PO₄, 0.5 g/L of NaCl, 1 g/L of NH₄Cl, 5 g/L of glucose, 15 g/L of agar) containing 12.5 mg/L of tetracycline, a succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, was constructed.

[0161]

<1-2-2> Evaluation of effect of yeeA gene amplification in *P. ananatis* succinic acid-producing strain

The plasmid containing the yeeA gene obtained by the screening was introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, to thereby construct a yeeA gene-amplified strain (yeeA-amplified strain). pSTV28 was also introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, to thereby construct a control strain (vector control strain). These strains were each cultured with shaking at 34°C for 20.5 hours by using the MS 3% Sucrose for Succinate medium. After the culture, growth, consumed sugar amount, and accumulation amounts of α -KG and succinic acid in the medium were measured. The composition of the MS 3% Sucrose for Succinate medium is shown below.

[0162]

[Composition of MS 3% Sucrose for Succinate medium]

[Group A]

Sucrose	30 g/L
MgSO ₄ · 7H ₂ O	0.5 g/L

[Group B]

(NH ₄) ₂ SO ₄	5.0 g/L
KH ₂ PO ₄	2.0 g/L
Yeast extract	2.0 g/L
FeSO ₄ · 7H ₂ O	0.01 g/L
MnSO ₄ · 5H ₂ O	0.01 g/L

(adjusted to pH 6.5 with KOH)

[Group C]

CaCO₃ 20 g/L

The components of the groups A and B were each sterilized in an autoclave at 115°C for 10 minute, the component of Group C was sterilized with hot air at 180°C for 3 hours, then they were mixed, and tetracycline and chloramphenicol were added to the mixture at 12.5 mg/L and 25 mg/L, respectively.

[0163]

The results are shown in Table 1. The data are shown in the table as average $\pm$ standard deviation of the results of the culture performed in triplicate for each strain. The succinic acid accumulation amount of the yeeA-amplified strain was almost equivalent to that of the control strain. The α -KG accumulation amount of the yeeA-amplified strain was higher than that of the control strain. These results suggest that YeeA recognizes not only succinic acid but also α -KG as a substrate.

[0164]

[Table 1]

Table 1: Effect of yeeA gene amplification in *P. ananatis* succinic acid-producing strain SC17(0) Δ sdhA/RSFPP

	OD620nm (x1/51)	α -KG (g/L)	Succinate (g/L)	Consumed Sucrose (g/L)
Vector control strain	0.334 $\pm$ 0.005	0.3 $\pm$ 0.0	9.6 $\pm$ 0.1	31.4 $\pm$ 0.0
yeeA-Amplified strain	0.345 $\pm$ 0.021	6.5 $\pm$ 1.5	9.2 $\pm$ 0.6	31.4 $\pm$ 0.0

[0165]

<1-3> Evaluation of effect of yeeA gene amplification in *P.*

79

ananatis α -KG-producing strain

<1-3-1> Construction of α -KG-producing bacterium, *P. ananatis* SC17sucA/RSFPP strain

[0166]

The RSFPP plasmid was introduced into the *P. ananatis* SC17sucA strain (WO2005/085419), to thereby construct an α -KG-producing bacterium, SC17sucA/RSFPP strain. The SC17sucA strain was a *sucA* gene-deficient strain of the *P. ananatis* SC17 strain, and is also referred to as AJ417 strain. The SC17sucA strain (AJ417 strain) was deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 26, 2004, and assigned an accession number of FERM BP-08646.

[0167]

<1-3-2> Evaluation of effect of *yeeA* gene amplification in *P. ananatis* α -KG-producing strain

The plasmid containing the *yeeA* gene obtained by the screening was introduced into the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct a *yeeA* gene-amplified strain (*yeeA*-amplified strain). pSTV28 was also introduced into the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct a control strain (vector control strain).

[0168]

These strains were each cultured with shaking at 34°C for

23 hours by using the MS 3% Sucrose for Succinate medium supplemented with 200 mg/L each of Lys, Met, and DAP. After the culture, growth, consumed sugar amount, and accumulation amounts of Glu, α -KG, and acetic acid in the medium were measured. The results are shown in Table 2. The data are shown in the table as average $\pm$ standard deviation of the results of the culture performed in quadruplicate for each strain. The Glu accumulation amount of the yeeA-amplified strain decreased, and the α -KG and acetic acid accumulation amounts of the yeeA-amplified strain increased, as compared with those of the control strain. On the basis of these results, it is considered that the α -KG concentration in the cells was reduced due to the amplification of the yeeA gene, and thereby conversion of α -KG into Glu was suppressed.

[0169]

[Table 2]

Table 2: Effect of yeeA gene amplification in *P. ananatis* α -KG-producing strain SC17sucA/RSFPP

	OD620nm (x1/51)	Glu (g/L)	Consumed Sucrose (g/L)	α -KG (g/L)	Acetate (g/L)
Vector control strain	0.311 $\pm$ 0.005	2.7 $\pm$ 0.1	31 $\pm$ 0	14 $\pm$ 0.2	0.1 $\pm$ 0
yeeA-Amplified strain	0.311 $\pm$ 0.007	0.6 $\pm$ 0	31 $\pm$ 0	15.2 $\pm$ 0.5	0.9 $\pm$ 0.1

[0170]

<1-4> Verification of effect of amplification of yeeA gene alone

The plasmid containing the yeeA gene obtained by the screening also contained genes around the yeeA gene. Therefore, it was determined whether the same effect as that obtained by introducing the aforementioned plasmid could also be obtained by amplification of the yeeA gene alone.

[0171]

PCR was performed by using the primers shown as SEQ ID NOS: 29 and 30, and the chromosomal DNA of the *P. ananatis* AJ13355 strain (FERM BP-6614) as the template to obtain a DNA fragment containing the *yeeA* gene. The obtained DNA fragment was treated with *Hind*III and *Kpn*I, and then inserted into pMW219 at the site for these restriction enzymes to obtain a *yeeA* gene expression plasmid pMW-PanyeeA. pMW-PanyeeA was introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, and the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct *yeeA* gene-amplified strains. Further, pMW218 was also introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, and the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct control strains.

[0172]

These strains were each cultured with shaking at 34°C for 18.5 hours using the MS 3% Sucrose for Succinate medium supplemented with 200 mg/L each of Lys, Met, and DAP. After the culture, growth, consumed sugar amount, and accumulation amounts of α -KG and succinic acid in the medium were measured. The results are shown in Table 3. The data are shown in the table as average $\pm$ standard deviation of the results of the culture performed in triplicate for each strain. When the *yeeA* gene alone was amplified in the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, the accumulation amount of α -KG increased, and the accumulation amount of succinic acid did not substantially change, as in the case where the plasmid obtained by the screening was introduced. When the *yeeA* gene alone was amplified also in the α -KG-producing bacterium,

82

SC17sucA/RSFPP strain, the accumulation amount of α -KG increased as in the case where the plasmid obtained by the screening was introduced. On the basis of these results, it was confirmed again that the *yeeA* gene is a dicarboxylic acid efflux carrier gene.

[0173]

[Table 3]

Table 3: Effect of amplification of *yeeA* gene alone in *P. ananatis* succinic acid-producing strain and α -KG-producing strain

	OD620nm (x1/51)	Consumed Sucrose (g/L)	α -KG (g/L)	Succinate (g/L)
SC17(0) Δ sdhA/RSFPP pMW218	0.274 $\pm$ 0.007	32.3 $\pm$ 0.1	0.8 $\pm$ 0.1	10 $\pm$ 0.2
SC17(0) Δ sdhA/RSFPP pMW-PanyeeA	0.222 $\pm$ 0.021	33.1 $\pm$ 0	5.4 $\pm$ 0.2	9.2 $\pm$ 0.3
SC17sucA/RSFPP pMW218	0.242 $\pm$ 0.002	31.7 $\pm$ 0.4	11.2 $\pm$ 0.1	0.1 $\pm$ 0
SC17sucA/RSFPP pMW-PanyeeA	0.28 $\pm$ 0.003	33.3 $\pm$ 0.1	17.2 $\pm$ 0.6	0.1 $\pm$ 0

[0174]

<Example 2> Evaluation of effect of *ynfM* gene amplification in *P. ananatis* succinic acid-producing strain and α -KG-producing strain

In this example, effect of *ynfM* gene amplification on dicarboxylic acid production was evaluated by using succinic acid-producing strain derived from the *P. ananatis* SC17(0) strain and α -KG-producing strain derived from the *P. ananatis* SC17 strain as the hosts.

[0175]

PCR was performed by using the primers shown as SEQ ID NOS: 31 and 32, and the chromosomal DNA of the *P. ananatis* AJ13355 strain (FERM BP-6614) as the template to obtain a DNA fragment containing the *ynfM* gene. The obtained DNA fragment was treated with *EcoRI* and *PstI*, and then inserted into pMW218

at the site for these restriction enzymes to obtain a *ynfM* gene expression plasmid pMW-PanynfM. pMW-PanynfM was introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, and the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct *ynfM* gene-amplified strains. Further, pMW218 was also introduced into the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, and the α -KG-producing bacterium, SC17sucA/RSFPP strain, to thereby construct control strains. [0176]

These strains were each cultured with shaking at 34°C for 18.5 hours by using the MS 3% Sucrose for Succinate medium supplemented with 200 mg/L each of Lys, Met, and DAP. After the culture, growth, consumed sugar amount, and accumulation amounts of α -KG and succinic acid in the medium were measured. The results are shown in Table 4. The data are shown in the table as average $\pm$ standard deviation of the results of the culture performed in triplicate for each strain. When the *ynfM* gene was amplified in the succinic acid-producing bacterium, SC17(0) Δ sdhA/RSFPP strain, the accumulation amount of α -KG slightly increased, and the accumulation amount of succinic acid did not significantly change. When the *ynfM* gene was amplified in the α -KG-producing bacterium, SC17sucA/RSFPP strain, the accumulation amount of α -KG markedly increased.

[0177]

[Table 4]

Table 4: Effect of amplification of *ynfM* gene in *P. ananatis* succinic acid-producing strain and α -KG-producing strain

	OD620nm (x1/51)	Consumed Sucrose (g/L)	α -KG (g/L)	Succinate (g/L)
SC17(0) Δ sdhA/RSFPP pMW218	0.274 $\pm$ 0.007	32.3 $\pm$ 0.1	0.8 $\pm$ 0.1	10 $\pm$ 0.2
SC17(0) Δ sdhA/RSFPP pMW-PanynfM	0.214 $\pm$ 0.014	33.3 $\pm$ 0.1	2.5 $\pm$ 0.1	9.1 $\pm$ 0.2
SC17sucA/RSFPP pMW218	0.242 $\pm$ 0.002	31.7 $\pm$ 0.4	11.2 $\pm$ 0.1	0.1 $\pm$ 0
SC17sucA/RSFPP pMW-PanynfM	0.28 $\pm$ 0.007	33.1 $\pm$ 0.1	15.4 $\pm$ 0.3	0.1 $\pm$ 0

[0178]

<Example 3> Evaluation of effect of *yeeA* gene and *ynfM* gene amplification in *P. ananatis* malic acid-producing strain

In this example, effects of *yeeA* gene amplification and *ynfM* gene amplification on dicarboxylic acid production were evaluated by using a malic acid-producing strain derived from *P. ananatis* SC17(0) strain as the host.

[0179]

<3-1> Construction of malic acid-producing bacterium, *P. ananatis* SC17(0) Δ m5/RSFPP strain

P. ananatis contains three enzymes and five genes for them, malate dehydrogenase (*mdh*), malate-quinone oxidoreductase (*mgo1* and *mgo2*), and malic enzyme (*sfcA* and *maeB*), as candidates for enzymes and genes of the malic acid decomposition system. Therefore, a multiple deficient strain for these five genes was constructed first.

[0180]

PCR was performed by using the primers shown as SEQ ID NOS: 33 and 34, and pMW118-attL-Km^r-attR as the template to amplify a DNA fragment for disruption of the *mdh* gene. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA

85

fragment to obtain SC17(0) Δ mdh::Km strain in which the *mdh* gene was replaced with the kanamycin (Km) resistance gene. The Km resistance gene was removed from this strain to obtain SC17(0) Δ mdh deficient in the *mdh* gene.

[0181]

PCR was performed by using the primers shown as SEQ ID NOS: 35 and 36, and pMW118-attL-Tet^r-attR (WO2005/010175) as the template to amplify a DNA fragment for disruption of the *mgo1* gene. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ mgo::Tet strain in which the *mgo1* gene was replaced with the tetracycline (Tet) resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ mdh strain to obtain SC17(0) Δ mdh Δ mgo::Tet strain.

[0182]

PCR was performed by using the primers shown as SEQ ID NOS: 37 and 38, and pMW118-attL-Km^r-attR as the template to amplify a DNA fragment for disruption of the *mgo2* gene. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ mgo2::Km strain in which the *mgo2* gene was replaced with the Km resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ mdh Δ mgo::Tet strain to obtain SC17(0) Δ mdh Δ mgo::Tet Δ mgo2::Km strain. The drug resistance genes were removed from this strain to obtain SC17(0) Δ m3 strain deficient in the *mdh*, *mgo1*, and *mgo2* genes.

[0183]

PCR was performed by using the primers shown as SEQ ID NOS: 39 and 40, and pMW118-attL-Km^r-attR as the template to amplify a DNA fragment for disruption of the *sfcA* gene. The

SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ sfcA::Km strain in which the *sfcA* gene was replaced with the Km resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ m3 strain to obtain SC17(0) Δ m3 Δ sfcA::Km strain.

[0184]

PCR was performed by using the primers shown as SEQ ID NOS: 41 and 42, and pMW118-attL-Tet^r-attR as the template to amplify a DNA fragment for disruption of the *maeB* gene. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ maeB::Tet strain in which the *maeB* gene was replaced with the Tet resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ m3 Δ sfcA::Km strain to obtain SC17(0) Δ m3 Δ sfcA::Km Δ maeB::Tet strain. The drug resistance genes were removed from this strain to obtain SC17(0) Δ m5 strain deficient in the *mdh*, *mqo1*, *mgo2*, *sfcA*, and *maeB* genes.

[0185]

The RSFPP plasmid was introduced into the SC17(0) Δ m5 strain, to thereby construct a malic acid-producing bacterium, SC17(0) Δ m5/RSFPP strain.

[0186]

These strains were each cultured with shaking at 34°C for 19 hours by using the MS 3% Sucrose for Succinate medium. After the culture, growth, consumed sugar amount, and accumulation amounts of organic acids in the medium were measured. The results are shown in Table 5. The data are shown in the table as average $\pm$ standard deviation. In the RSFPP-introduced strain, the acetic acid accumulation amount decreased, and growth, sugar consumption amount, and malic acid accumulation

amount increased, as compared with the strain not introduced with RSFPP.

[0187]

[Table 5]

Table 5: Malic acid-producing ability of malic acid decomposition system enzyme-multiple deficient *P. ananatis* strain

	OD620nm (x1/51)	Consumed Sucrose (g/L)	α -KG (g/L)	Malate (g/L)	Succinate (g/L)	Fumarate (g/L)	Acetate (g/L)
SC17(0) Δ m5	0.045 $\pm$ 0.003	3.6 $\pm$ 0.1	0.1 $\pm$ 0.1	0.5 $\pm$ 0.1	0.1 $\pm$ 0.1	0 $\pm$ 0	1.3 $\pm$ 0.2
SC17(0) Δ m5/RSFPP	0.068 $\pm$ 0.01	6.1 $\pm$ 0.3	0 $\pm$ 0.1	2.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.6 $\pm$ 0

[0188]

<3-2> Evaluation of effect of *yeeA* gene and *ynfM* gene amplification in *P. ananatis* malic acid-producing bacterium
pMW-PanyeeA and pMW-PanynfM were each introduced into the malic acid-producing bacterium, SC17(0) Δ m5/RSFPP strain, to thereby construct a *yeeA* gene-amplified strain and *ynfM* gene-amplified strain, respectively. pMW218 was also introduced into the malic acid-producing bacterium, SC17(0) Δ m5/RSFPP strain, to thereby construct a control strain.

[0189]

These strains were each cultured with shaking at 34°C for 18.5 hours by using the MS 3% Sucrose for Succinate medium. After the culture, growth, consumed sugar amount, and accumulation amounts of organic acids in the medium were measured. The results are shown in Table 6. The data are shown in the table as average $\pm$ standard deviation. In the *yeeA* gene-amplified strain and the *ynfM* gene-amplified strain, growth, consumed sugar amount, and accumulation amounts of various dicarboxylic acids such as malic acid markedly increased as compared with the control strain. Marked effect

was obtained especially in the *yeeA*-amplified strain. These results strongly suggested that these dicarboxylic acid efflux carriers are transporters for excreting various dicarboxylic acids.

[0190]

[Table 6]

Table 6: Effect of amplification of *yeeA* gene and *ynfM* gene in *P. ananatis* malic acid-producing bacterium

	OD620nm (x1/51)	Consumed Sucrose (g/L)	α -KG (g/L)	Malate (g/L)	Succinate (g/L)	Fumarate (g/L)	Acetate (g/L)
pMW218	0.054 $\pm$ 0.002	4.5 $\pm$ 0	0.9 $\pm$ 0	1.7 $\pm$ 0	0.1 $\pm$ 0	0.3 $\pm$ 0	0.5 $\pm$ 0
pMW-PanyeeA	0.265 $\pm$ 0.013	31.7 $\pm$ 0	1.6 $\pm$ 0.2	6.9 $\pm$ 1.3	5.2 $\pm$ 1.3	0.7 $\pm$ 0.1	0.1 $\pm$ 0
pMW-PanynfM	0.122 $\pm$ 0.005	10.5 $\pm$ 0.3	0.9 $\pm$ 0	3.2 $\pm$ 0.1	1 $\pm$ 0	0.6 $\pm$ 0	0.2 $\pm$ 0

[0191]

<Example 4> Evaluation of effect of *yeeA* gene and *ynfM* gene amplification in *P. ananatis* itaconic acid-producing strain

In this example, effects of *yeeA* gene amplification and *ynfM* gene amplification on itaconic acid production were evaluated by using an itaconic acid-producing strain derived from *P. ananatis* SC17(0) strain as the host.

[0192]

<4-1> Construction of itaconic acid-producing bacterium, *P. ananatis* ITC01 strain

Itaconic acid is generated by decarboxylation of cis-aconitic acid, which is an intermediate of the citrate-isocitrate conversion in the TCA cycle. The decarboxylation of cis-aconitic acid is catalyzed by cis-aconitate decarboxylase (CAD). Therefore, CAD was enhanced, and isocitrate dehydrogenase (ICDH) and isocitrate lyase (ICL), which are isocitric acid decomposition system enzymes, were blocked.

[0193]

PCR was performed by using the primers shown as SEQ ID NOS: 43 and 44, and pMW118-attL-Km^r-attR as the template to amplify a DNA fragment for disruption of the *icd* gene. The *icd* gene is a gene encoding isocitrate dehydrogenase (ICDH). The SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ *icd*::Km strain in which the *icd* gene was replaced with the Km resistance gene. The Km resistance gene was removed from this strain to obtain SC17(0) Δ *icd* deficient in the *icd* gene.

[0194]

PCR was performed by using the primers shown as SEQ ID NOS: 45 and 46, and pMW118-attL-Km^r-attR as the template to amplify a DNA fragment for disruption of the *sdhA* gene. The *sdhA* gene is a gene encoding succinate dehydrogenase. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA fragment to obtain SC17(0) Δ *sdhA*::Km strain in which the *sdhA* gene was replaced with the Km resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ *icd* strain to obtain SC17(0) Δ *icd* Δ *sdhA*::Km strain.

[0195]

PCR was performed by using the primers shown as SEQ ID NOS: 47 and 48, and pMW118-attL-Tet^r-attR as the template to amplify a DNA fragment for disruption of the *aceBAK* genes. This DNA fragment had a sequence complementary to an internal sequence of the *aceB* gene, and a sequence complementary in an internal sequence of the *aceK* gene, at the respective ends. The *aceB* gene is a gene encoding malate synthase. The *aceA* gene is a gene encoding isocitrate lyase. The *aceK* gene is a gene encoding isocitrate dehydrogenase kinase/phosphatase. The SC17(0)/RSFRedTER strain was transformed with the obtained DNA

fragment to obtain SC17(0) Δ aceBAK::Tet strain in which the aceB-aceA-aceK genes were replaced with the Tet resistance gene. The chromosome was extracted from this strain, and used to transform the SC17(0) Δ icd Δ sdhA::Km strain to obtain SC17(0) Δ icd Δ sdhA::Km Δ aceBAK::Tet strain. The drug resistance genes were removed from this strain to obtain SC17(0) Δ icd Δ sdhA Δ aceBAK strain deficient in the *icd*, *sdhA*, and *aceBAK* genes.

[0196]

As for the cis-aconitate decarboxylase (CAD) gene, a nucleotide sequence optimized for the codon usage of *E. coli* (SEQ ID NO: 49) was designed on the basis of the nucleotide sequence of the CAD gene of *Aspergillus terreus*, and used. Optimization and synthesis of the nucleotide sequence of the CAD gene were entrusted to Genscript, and pSTV-CAD having the CAD gene introduced into the *HindIII*-*KpnI* site of pSTV28 was obtained. The pSTV-CAD plasmid and the RSFPP plasmid were introduced into the SC17(0) Δ icd Δ sdhA Δ aceBAK strain to construct an itaconic acid-producing bacterium, ITC01 (SC17(0) Δ icd Δ sdhA Δ aceBAK/RSFPP+pSTV-CAD) strain. The pSTV28 plasmid and the RSFPP plasmid were introduced into the SC17(0) Δ icd Δ sdhA Δ aceBAK strain to construct a control strain (-CAD). These strains were each cultured with shaking at 34°C by using the MS 3% Sucrose for Succinate medium supplemented with glutamic acid at a final concentration of 5 g/L, and growth, and accumulation amount of itaconic acid in the medium were measured. The results are shown in Fig. 1. Whereas the control strain (-CAD) did not produce itaconic acid at all, the ITC01 strain accumulated about 9 g/L of itaconic acid.

[0197]

<4-2> Evaluation of effect of *yeeA* gene and *ynfM* gene amplification in *P. ananatis* itaconic-acid-producing strain

pMW-PanyeeA and pMW-PanynfM were each introduced into the itaconic acid-producing bacterium, ITC01 strain, to thereby construct a *yeeA* gene-amplified strain and *ynfM* gene-amplified strain, respectively. pMW219 was also introduced into the itaconic acid-producing bacterium, ITC01 strain, to thereby construct a control strain.

[0198]

These strains were each cultured with shaking at 34°C for 48 hours by using the MS 3% Sucrose for Succinate medium supplemented with glutamic acid at a final concentration of 5 g/L. After the culture, growth, consumed sugar amount, amount of remaining glutamic acid, and accumulation amount of itaconic acid in the medium were measured. The results are shown in Table 7. The data are shown in the table as average $\pm$ standard deviation. In the *yeeA* gene-amplified strain and the *ynfM* gene-amplified strain, consumed sugar amount increased, and accumulation amount of itaconic acid also increased, as compared with the control strain. With the *yeeA* gene-amplified strain and the *ynfM* gene-amplified strain, the added Glu was totally consumed, and most of it was converted into succinic acid (data are not shown).

[0199]

[Table 7]

Table 7: Effect of amplification of *yeeA* gene and *ynfM* gene in *P. ananatis* itaconic acid-producing strain derived from SC17 (0)

	OD620nm (x1/51)	Glu (g/L)	Consumed sucrose (g/L)	Itaconate (g/L)	Itaconate yield (%)
ITC01/pMW219	0.081 $\pm$ 0.004	3.2 $\pm$ 0	11.2 $\pm$ 0.5	5.3 $\pm$ 0.4	47.5 $\pm$ 3.1
ITC01/pMW-PanyeeA	0.048 $\pm$ 0.003	0 $\pm$ 0	34.5 $\pm$ 0.1	18.1 $\pm$ 0.1	52.5 $\pm$ 0.2
ITC01/pMW-PanynfM	0.081 $\pm$ 0.014	0 $\pm$ 0	32.5 $\pm$ 0.6	14 $\pm$ 2	43.1 $\pm$ 5.7

[0200]

<Example 5> Evaluation of effect of *yjjPB* gene amplification on succinic acid production in *E. aerogenes*

In this example, effects of *yjjPB* gene amplification on succinic acid production was evaluated by using a succinic acid-producing strain derived from the *Enterobacter aerogenes* AJ110637 strain (FERM BP-10955) as the host. The AJ110637 strain (FERM BP-10955) was deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on August 22, 2007, and assigned an accession number of FERM BP-10955.

[0201]

<5-1> Construction of *E. aerogenes* ES06 strain

The *poxB* gene on the genome of the *E. aerogenes* ES04 strain (US20100297716A1) constructed from the *Enterobacter aerogenes* AJ110637 (FERM BP-10955) strain was replaced with the *pckA* gene derived from the *Actinobacillus succinogenes* 130Z strain to construct *Enterobacter aerogenes* ES06 strain. The procedure is shown below.

[0202]

Construction of $\lambda attL$ -Km^r- $\lambda attR$ -Ptac-*pckA* gene fragment

The total nucleotide sequence of the genomic DNA of the *Actinobacillus succinogenes* 130Z strain (ATCC 55618) (GenBank Accession No. CP000746) has already been opened to public, and

the gene encoding phosphoenolpyruvate carboxykinase (gene name *pckA*, registration number Asuc_0221) has also been elucidated. The nucleotide sequence of the *pckA* gene of the *Actinobacillus succinogenes* 130Z strain is shown as SEQ ID NO: 50, and the amino acid sequence of the phosphoenolpyruvate carboxykinase encoded by this gene is shown as SEQ ID NO: 51. By using the genomic DNA of the *Actinobacillus succinogenes* 130Z strain as the template, and the primers shown as SEQ ID NOS: 52 and 53 designed on the basis of the aforementioned nucleotide sequence, PCR was performed (TaKaRa Prime Star (registered trademark), 94°C for 10 seconds, 54°C for 20 seconds, 72°C for 90 seconds, 30 cycles) to obtain a DNA fragment containing the ORF region of *pckA*. PCR was also performed by using a DNA fragment containing λ attL-Km^r- λ attR-Ptac (WO2008/090770A1) as the template, and the primers shown as SEQ ID NOS: 54 and 55 (TaKaRa Prime Star (registered trademark), 94°C for 10 seconds, 54°C for 20 seconds, 72°C for 90 seconds, 30 cycles) to obtain a DNA fragment containing λ attL-Km^r- λ attR-Ptac. Then, by using the DNA fragment containing the ORF region of *pckA* and the DNA fragment containing λ attL-Km^r- λ attR-Ptac as the template, and the primers shown as SEQ ID NOS: 53 and 54, PCR was performed (TaKaRa Prime Star (registered trademark), 94°C for 10 seconds, 54°C for 20 seconds, 72°C for 180 seconds, 35 cycles) to obtain a λ attL-Km^r- λ attR-Ptac-*pckA* gene fragment having sequences for recombination with the gene encoding pyruvate oxidase (gene name, *poxB*) at the both ends.

[0203]

Construction of ES04/RSFRedTER strain

The ES04 strain (US20100297716A1) was cultured overnight in the LB liquid medium. Then, the culture broth (100 μ L) was

inoculated into fresh LB liquid medium (4 mL), and shaking culture was performed at 34°C for 3 hours. The cells were collected, washed 3 times with 10% glycerol, and used as competent cells. RSFRedTER (WO2008/090770A1) was introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . The cells were cultured for 2 hours in the SOC medium (20 g/L of Bacto tryptone, 5 g/L of yeast extract, 0.5 g/L of NaCl, 10 g/L of glucose), then applied to the LB agar medium containing 40 mg/L of chloramphenicol, and cultured for 16 hours. As a result, a transformant showing chloramphenicol resistance was obtained, and designated as ES04/RSFRedTER strain.

[0204]

Construction of ES04 Δ poxB:: λ attL-Km^r- λ attR-Ptac-pckA strain

The ES04/RSFRedTER strain was cultured overnight in the LB liquid medium. Then, the culture broth (1 mL) was inoculated into LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 40 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, washed 3 times with 10% glycerol, and used as competent cells. The amplified λ attL-Km^r- λ attR-Ptac-pckA gene fragment was purified by using Wizard PCR Prep DNA Purification System (Promega), and introduced into the competent cells by electroporation. The cells were cultured for 2 hours in the SOC medium, then applied to the LB agar medium containing 50 mg/L of kanamycin, and cultured for 16 hours. A colony that appeared was purified on the same medium, and used together with the primers shown as SEQ ID NOS: 56 and 57 to

perform colony PCR (TaKaRa Speed Star (registered trademark), 92°C for 10 seconds, 56°C for 10 seconds, 72°C for 30 seconds, 40 cycles) and thereby confirm that the *poxB* gene on the genome had been replaced with the $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA* gene. The obtained strain was applied to the LB agar medium containing 10% sucrose and 1 mM IPTG to remove the RSFRedTER plasmid and thereby obtain ES04 Δ *poxB*:: $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA* strain. [0205]

Removal of kanamycin resistance gene from
ES04 Δ *poxB*:: $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA* strain

In order to remove the kanamycin resistance gene from the ES04 Δ *poxB*:: $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA* strain, the RSF-int-xis plasmid (US20100297716A1) was used. RSF-int-xis was introduced into the ES04 Δ *poxB*:: $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA* strain by electroporation, and the strain was applied to the LB agar medium containing 40 mg/L of chloramphenicol, and cultured at 30°C to obtain ES04 Δ *poxB*:: $\lambda attL$ -Km^r- $\lambda attR$ -*Ptac*-*pckA*/RSF-int-xis strain. The obtained strain harboring the plasmid was purified on the LB agar medium containing 40 mg/L of chloramphenicol and 1 mM IPTG to obtain a plurality of single colonies. Then, the colonies were applied on the medium containing 50 mg/L of kanamycin, and cultured overnight at 37°C. A strain confirmed to be a strain from which the kanamycin resistance gene was removed by confirming that it could not grow was obtained. Then, in order to remove the RSF-int-xis plasmid from the obtained strain, it was applied to the LB agar medium containing 10% sucrose and 1 mM IPTG, and cultured overnight at 37°C. A strain showing chloramphenicol susceptibility among those of the colonies that appeared was designated as ES06 strain.

[0206]

<5-2> Construction of *E. aerogenes* ES06 Δ yeeA strain

The ES06 Δ yeeA strain was constructed by the λ -red method. Specifically, PCR was performed by using the primers shown as SEQ ID NOS: 58 and 59, and pMW118-attL-Km^r-attR as the template to amplify a fragment having sequences of 50 bp complementary to an internal sequence of the yeeA gene of *Enterobacter aerogenes* at the both ends, and the kanamycin resistance gene between the attL and attR sequences of λ phage. ES06/RSFRedTER strain obtained by introducing RSFRedTER into the ES06 strain by electroporation was cultured overnight in the LB liquid medium, the culture broth (1 mL) was inoculated into the LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. The amplified PCR fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . By selection on the LB agar medium containing 40 mg/L of kanamycin, ES06 Δ yeeA::Km strain was obtained. The obtained strain was applied to the LB agar medium containing the M9 components (17.1 g/L of Na₂HPO₄·12H₂O, 3 g/L of KH₂PO₄, 0.5 g/L of NaCl, and 1 g/L of NH₄Cl), 10% sucrose, and 1 mM IPTG to obtain a strain from which the RSFRedTER plasmid was removed. Into this strain, the pMW-intxis-sacB(Cm) plasmid was introduced by electroporation, and by selection on the LB agar medium containing 25 mg/L of chloramphenicol,

ES06ΔyeeA::Km/pMW-intxis-sacB(Cm) strain was obtained. This strain was purified on the LB agar medium, then replicated on the LB agar medium containing 40 mg/L of kanamycin, and a strain that became kanamycin and chloramphenicol-sensitive was designated as ES06ΔyeeA strain.

[0207]

<5-3> Construction of *E. aerogenes* ES06ΔyeeAΔynfM strain

The ES06ΔyeeAΔynfM strain was constructed by the aforementioned λ-red method from the ES06ΔyeeA strain. Specifically, PCR was performed by using the primers shown as SEQ ID NOS: 60 and 61, and pMW118-attL-Km^r-attR as the template to amplify a fragment having sequences of 50 bp complementary to an internal sequence of the *ynfM* gene of *Enterobacter aerogenes* at the both ends, and the kanamycin resistance gene between the *attL* and *attR* sequences of λ phage.

ES06ΔyeeA/RSFRedTER strain obtained by introducing RSFRedTER into the ES06ΔyeeA strain by electroporation was cultured overnight in the LB liquid medium, the culture broth (1 mL) was inoculated into the LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. The amplified PCR fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μF, and resistance of 200 Ω. By selection on the LB agar medium containing 40 mg/L of kanamycin, ES06ΔyeeAΔynfM::Km strain was obtained. The obtained strain was applied to the

LB agar medium containing the M9 components (17.1 g/L of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 3 g/L of KH_2PO_4 , 0.5 g/L of NaCl, and 1 g/L of NH_4Cl), 10% sucrose, and 1 mM IPTG to obtain a strain from which the RSFRedTER plasmid was removed. To this strain, the pMW-intxis-sacB(Cm) plasmid was introduced by electroporation, and by selection on the LB agar medium containing 25 mg/L of chloramphenicol, ES06 Δ yeeA Δ ynfM::Km/pMW-intxis-sacB(Cm) strain was obtained. This strain was purified on the LB agar medium, then replicated on the LB agar medium containing 40 mg/L of kanamycin, and a strain that became kanamycin-sensitive was designated as ES06 Δ yeeA Δ ynfM strain.

[0208]

<5-4> Construction of *E. aerogenes* *sdhA*-disrupted strain

ES06 Δ sdhA strain deficient in the *sdhA* gene encoding a subunit of succinate dehydrogenase was constructed from the ES06 strain by the aforementioned λ -red method. Specifically, PCR was performed by using the primers shown as SEQ ID NOS: 62 and 63, and pMW118-attL-Km^r-attR as the template to amplify a fragment having sequences of 50 bp complementary to an internal sequence of the *sdhA* gene of *Enterobacter aerogenes* at the both ends, and the kanamycin resistance gene between the attL and attR sequences of λ phage. The ES06/RSFRedTER strain was cultured overnight in the LB liquid medium, the culture broth (1 mL) was inoculated into the LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. The amplified PCR fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The

electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . By selection on the LB agar medium containing 40 mg/L of kanamycin, and 20 mM disodium malate, ES06 Δ sdhA::Km strain was obtained. The obtained strain was applied to the LB agar medium containing the M9 components (17.1 g/L of Na₂HPO₄·12H₂O, 3 g/L of KH₂PO₄, 0.5 g/L of NaCl, and 1 g/L of NH₄Cl), 10% sucrose, and 1 mM IPTG to obtain a strain from which the RSFRedTER plasmid was removed. To this strain, the pMW-intxis-sacB(Cm) plasmid was introduced by electroporation, and by selection on the LB agar medium containing 25 mg/L of chloramphenicol, and 20 mM disodium malate, ES06 Δ sdhA::Km/pMW-intxis-sacB(Cm) strain was obtained. This strain was purified on the LB agar medium containing 20 mM disodium malate, and then replicated on the LB agar medium containing 40 mg/L of kanamycin and 20 mM disodium malate, and a strain that became kanamycin-sensitive was designated as ES06 Δ sdhA strain. Strains deficient in the *sdhA* gene were also derived from the ES06 Δ yeeA and ES06 Δ yeeA Δ ynfM strains in a similar manner, and designated as ES06 Δ sdhA Δ yeeA strain and ES06 Δ sdhA Δ yeeA Δ ynfM strain, respectively.

[0209]

<5-5> Construction of pSTV28-AeyjjPB plasmid

pSTV28-AeyjjPB plasmid is a plasmid consisting of pSTV vector carrying the *yjjPB* genes derived from the *E. aerogenes* AJ110637 strain (FERM BP-10955). Specifically, it was constructed by the following method.

[0210]

Primers S3 and S4 (SEQ ID NOS: 64 and 65) for amplifying the *yjjPB* genes derived from *E. aerogenes* AJ110637 strain were

100

designed. PCR was performed by using these primers and the genome of the *E. aerogenes* AJ110637 strain as the template to obtain a DNA fragment containing the *yjjPB* genes. The obtained DNA fragment was inserted into the pSTV28 vector treated with *Bam*HI and *Pst*I using In Fusion HD Cloning Kit (Clontech). Competent cells of *Escherichia coli* JM109 (Takara Shuzo) were transformed with this DNA, applied to the LB agar medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 25 μ g/mL of Cm, and cultured overnight. Then, white colonies that appeared were picked up, and subjected to single colony isolation to obtain transformants. Plasmids were extracted from the obtained transformants, and a plasmid in which the objective PCR product was inserted was designated as pSTV28-AeyjjPB.

[0211]

<5-6> Construction of pSTV28-AeyjjP plasmid

pSTV28-AeyjjP plasmid is a plasmid consisting of pSTV vector carrying the *yjjP* gene derived from the *E. aerogenes* AJ110637 strain. Specifically, it was constructed by the following method.

[0212]

Primers S3 and S5 (SEQ ID NOS: 64 and 66) for amplifying the *yjjP* gene derived from the *E. aerogenes* AJ110637 strain were designed. PCR was performed by using these primers and the genome of the *E. aerogenes* AJ110637 strain as the template to obtain a DNA fragment containing the *yjjP* gene. The obtained DNA fragment was inserted into the pSTV28 vector treated with *Bam*HI and *Pst*I by using In Fusion HD Cloning Kit (Clontech). Competent cells of *Escherichia coli* JM109 (Takara Shuzo) were transformed with this DNA, applied to the LB agar medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 25 μ g/mL of Cm,

and cultured overnight. Then, white colonies that appeared were picked up, and subjected to single colony isolation to obtain transformants. Plasmids were extracted from the obtained transformants, and a plasmid in which the objective PCR product was inserted was designated as pSTV28-AeyjjP.

[0213]

<5-7> Construction of pSTV28-AePtac1000yjjB plasmid

pSTV28-AePtac1000yjjB plasmid is a plasmid consisting of the pSTV vector carrying the Ptac1000 promoter (SEQ ID NO: 67) and the *yjjB* gene derived from the *E. aerogenes* AJ110637 strain ligated together. Specifically, it was constructed by the following method.

[0214]

First, primers S6 and S7 (SEQ ID NOS: 68 and 69) for amplifying a Ptac1000 promoter fragment were designed. PCR was performed by using these primers and λ attL-Km^r- λ attR-Ptac (WO2008/090770A1) as the template to obtain a DNA fragment containing the Ptac1000 promoter. Then, primers S8 and S4 (SEQ ID NOS: 70 and 65) for amplifying the *yjjB* gene derived from the *E. aerogenes* AJ110637 strain were designed. PCR was performed by using these primers and the genome of the *E. aerogenes* AJ110637 strain as the template to obtain a DNA fragment containing the *yjjB* gene. The obtained both DNA fragments were inserted into the pSTV28 vector treated with *Bam*HI and *Pst*I by using In Fusion HD Cloning Kit (Clontech). Competent cells of *Escherichia coli* JM109 (Takara Shuzo) were transformed with this DNA, applied to the LB agar medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 25 μ g/mL of Cm, and cultured overnight. Then, white colonies that appeared were picked up, and subjected to single colony isolation to

obtain transformants. Plasmids were extracted from the obtained transformants, and a plasmid in which the objective PCR product was inserted was designated as pSTV28-AePtac1000yjjB.

[0215]

<5-8> Construction of *E. aerogenes* strains for evaluation

Into the ES06 Δ sdhA strain and ES06 Δ sdhA Δ yeeA Δ ynfM strain, the RSFPP plasmid, and pSTV28 vector, pSTV28-AeyjjPB plasmid, pSTV28-AeyjjP plasmid, or pSTV28-AePtac1000yjjB plasmid were introduced by electroporation, and the transformed cells were selected on the LB agar medium containing 12.5 mg/L of tetracycline, and 25 mg/L of chloramphenicol, and purified on the LB agar medium to obtain ES06 Δ sdhA/RSFPP+pSTV28 strain and ES06 Δ sdhA Δ yeeA Δ ynfM/RSFPP+pSTV28 strain as control strains, as well as ES06 Δ sdhA Δ yeeA Δ ynfM/RSFPP+pSTV28-AeyjjPB strain, ES06 Δ sdhA Δ yeeA Δ ynfM/RSFPP+pSTV28-AeyjjP strain, and ES06 Δ sdhA Δ yeeA Δ ynfM/RSFPP+pSTV28-AePtac1000yjjB strain as dicarboxylic acid efflux carrier gene-amplified strains.

[0216]

<5-9> Evaluation of succinic acid-producing ability

Then, succinic acid-producing abilities of these strains were evaluated. These strains were each cultured overnight at 34°C on an LBGM9 agar medium plate containing 12.5 mg/L of tetracycline and 25 mg/L of chloramphenicol. An appropriate amount of the obtained cells were inoculated into 5 mL of a succinic acid production medium contained in a test tube, and cultured at 34°C with shaking at 120 rpm. The composition of the succinic acid production medium is shown below.

[0217]

[Composition of succinic acid production medium]

103

[Group A]

Sucrose	30 g/L
MgSO ₄ · 7H ₂ O	0.5 g/L

[Group B]

(NH ₄) ₂ SO ₄	5.0 g/L
KH ₂ PO ₄	2.0 g/L
Yeast extract	2.0 g/L
FeSO ₄ · 7H ₂ O	0.01 g/L
MnSO ₄ · 5H ₂ O	0.01 g/L

(adjusted to pH 6.5 with KOH)

[Group C]

CaCO ₃	20 g/L
-------------------	--------

The components of the groups A and B were each sterilized in an autoclave at 115°C for 10 minute, the component of Group C was sterilized with hot air at 180°C for 3 hours, then they were mixed, and tetracycline and chloramphenicol were added to the mixture at 12.5 mg/L and 25 mg/L, respectively.

[0218]

The results of *yjjPB* gene amplification are shown in Table 8. As compared with the ES06Δ*sdhA*/RSFPP strain as the control, the succinic acid-producing ability decreased in the ES06Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP strain deficient in the *yeeA* gene and the *ynfM* gene. When the *yjjPB* genes were amplified in the ES06Δ*sdhA*Δ*yeeA*Δ*ynfM*/RSFPP strain as the host, succinic acid-producing ability increased to be higher than that of the ES06Δ*sdhA*/RSFPP strain not deficient in the *yeeA* gene and the *ynfM* gene. These results revealed that, by amplifying the *yjjPB* genes in *Enterobacter aerogenes*, succinic acid-producing ability thereof is improved.

[0219]

[Table 8]

Table 8: Influence of *yjjPB* gene amplification on succinic acid production in *E. aerogenes*

	OD620 nm (x1/51)	Consumed sugar (g/l)	Succinate (g/l)	Succinate (%)
ES06 Δ sdhA /RSFPP+pSTV28	0.27 $\pm$ 0.020	27 $\pm$ 2.1	7.9 $\pm$ 0.66	29.1 $\pm$ 0.82
ES06 Δ sdhA Δ yeeA Δ ynfM /RSFPP+pSTV28	0.21 $\pm$ 0.0070	24 $\pm$ 0.85	4.1 $\pm$ 0.11	17.2 $\pm$ 0.19
ES06 Δ sdhA Δ yeeA Δ ynfM /RSFPP+pSTV28-AeyjjPB	0.32 $\pm$ 0.12	25 $\pm$ 7.2	9.1 $\pm$ 1.9	37.7 $\pm$ 3.8

[0220]

<Example 6> Evaluation of effect of *ynfM* gene amplification on succinic acid production by coryneform bacterium

In this example, effect of *ynfM* gene amplification on succinic acid production was evaluated by using a succinic acid-producing strain derived from the *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) 2256 strain (ATCC 13869) as the host.

[0221]

<6-1> Construction of plasmid pVK9::PmsrA-*ynfM* for expression of *ynfM* gene

Plasmid pVK9::PmsrA-*ynfM* for expressing the *ynfM* gene derived from the *B. lactofermentum* 2256 strain was constructed by the following method.

[0222]

First, the *ynfM* gene was ligated with the promoter of the

105

msrA gene derived from the *B. lactofermentum* 2256 strain by crossover PCR. Specifically, PCR was performed by using the genomic DNA of the *B. lactofermentum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 71 and 72 as the primers to obtain a PCR product containing the promoter region of the *msrA* gene. Separately, PCR was performed by using the genomic DNA of the *B. lactofermentum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 73 and 74 as the primers to obtain a PCR product containing the ORF region of the *ynfM* gene. The sequences of SEQ ID NOS: 72 and 73 are partially complementary to each other. Then, the both PCR products were inserted into the pVK9 vector (WO2013/069634) treated with *Bam*HI and *Pst*I by using In Fusion HD Cloning Kit (Clontech). pVK9 is a shuttle vector of *Corynebacterium* bacteria and *E. coli*. With this DNA, competent cells of *Escherichia coli* JM109 (Takara Shuzo) were transformed, and applied to the LB agar medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 40 μ g/mL of kanamycin, and cultured overnight. Then, white colonies that appeared were picked up, and subjected to single colony isolation to obtain transformants. Plasmids were extracted from the obtained transformants, and a plasmid in which the target PCR product was inserted was designated as pVK9::PmsrA-ynfM.

[0223]

<6-2> Construction of *B. lactofermentum* 2256 Δ sdhA strain

<6-2-1> Construction of plasmid pBS4S Δ sdhA for deleting *sdhA* gene

PCR was performed by using the genomic DNA of the *B. lactofermentum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 75 and 76 as the primers to obtain a DNA

fragment containing the N-terminus side coding region of the *sdhA* gene. Separately, PCR was performed by using the genomic DNA of the *B. lactofermentum* 2256 strain as the template, and the synthetic DNAs of SEQ ID NOS: 77 and 78 as the primers to obtain a PCR product containing the C-terminus side coding region of the *sdhA* gene. The sequences of SEQ ID NOS: 76 and 77 are complementary to each other. Then, both the PCR products were mixed in substantially equimolar amounts, and used together with the synthetic DNAs of SEQ ID NOS: 79 and 80 as the primers to perform crossover PCR, and thereby obtain a DNA fragment for deleting *sdhA*. Then, this DNA fragment was treated with *Bam*HI, and inserted into the pBS4S vector (WO2007/046389) at the *Bam*HI site. Competent cells of *Escherichia coli* JM109 (Takara Shuzo) were transformed with this DNA, applied to the LB agar medium containing 100 μ M IPTG, 40 μ g/mL of X-Gal, and 40 μ g/mL of Km, and cultured overnight. Then, white colonies that appeared were picked up, and subjected to single colony isolation to obtain transformants. Plasmids were extracted from the obtained transformants, and a plasmid in which the objective PCR product was inserted was designated as p Δ *sdhA*56.

[0224]

<6-2-2> Construction of *B. lactofermentum* 2256 Δ *sdhA* strain

p Δ *sdhA*56 obtained above does not contain any region that enables autonomous replication thereof in cells of coryneform bacteria. Thus, when coryneform bacteria are transformed with this plasmid, a strain in which this plasmid is incorporated into the genome by homologous recombination appears as a transformant, although it occurs at an extremely low frequency. Therefore, p Δ *sdhA*56 was introduced into the *B. lactofermentum*

107

2256 strain by the electric pulse method. The cells were applied to the CM-Dex agar medium (5 g/L of glucose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean hydrolysate, 10 $\mu\text{g/L}$ of biotin, 15 g/L of agar, adjusted to pH 7.5 with NaOH) containing 25 $\mu\text{g/mL}$ of kanamycin, and cultured at 31.5°C. It was confirmed by PCR that the grown strain was a once-recombinant strain in which p $\Delta\text{sdhA}56$ was incorporated into the genome by homologous recombination. This once-recombinant strain had both the wild-type *sdhA* gene and the deletion type *sdhA* gene.

[0225]

The once-recombinant strain was cultured overnight in the CM-Dex liquid medium (having the same composition as that of the CM-Dex agar medium, with the proviso that 15 g/L of agar is omitted), and the culture broth was applied to the S10 agar medium (100 g/L of sucrose, 10 g/L of polypeptone, 10 g/L of yeast extract, 1 g/L of KH_2PO_4 , 0.4 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L of $\text{MnSO}_4 \cdot 4-5\text{H}_2\text{O}$, 3 g/L of urea, 1.2 g/L of soybean protein hydrolysate solution, 20 g/L of agar, adjusted to pH 7.5 with NaOH, and autoclaved at 120°C for 20 minutes), and cultured at 31.5°C. Among colonies that appeared, a strain that showed kanamycin susceptibility was purified on the CM-Dex agar medium. The genomic DNA was prepared from the purified strain, and used together with the synthetic DNAs of SEQ ID NOS: 75 and 78 as the primers to perform PCR and thereby confirm deletion of the wild-type *sdhA* gene, and the strain was designated as 2256 ΔsdhA strain.

[0226]

<6-3> Evaluation of succinic acid-producing ability

108

Into the 2256 Δ sdhA strain, the pVK9 plasmid and the pVK9::PamsrA-ynfM plasmid were each introduced by electroporation to obtain 2256 Δ sdhA/pVK9 strain as a control strain, and 2256 Δ sdhA/pVK9::PmsrA-ynfM as a *ynfM* gene-amplified strain.

[0227]

Then, succinic acid-producing abilities of these strains were evaluated. These strains were each cultured overnight at 31.5°C on a CM-Dex agar medium plate containing 25 mg/L of kanamycin. An appropriate amount of the obtained cells were inoculated into 5 mL of a succinic acid production medium contained in a test tube, and cultured at 31.5°C with shaking at 120 rpm. The composition of the succinic acid production medium is shown below.

[0228]

[Composition of succinic acid production medium]

Glucose	60 g/L
Ammonium sulfate	6 g/L
KH ₂ PO ₄	0.54 g/L
FeSO ₄ ·7H ₂ O	12 mg/L
MnSO ₄ ·5H ₂ O	12 mg/L
VB ₁	120 mg/L
Biotin	120 mg/L
Mameno	68 mg/L (in terms of total nitrogen)

The medium was adjusted to pH 7.0 with KOH, and sterilized at with 120°C for 20 minutes in an autoclave, and then MgCO₃ sterilized with hot air at 180°C for 3 hours or longer and left to cool was added to the medium at 50 g/L.

[0229]

The results are shown in Table 9. As compared with the 2256 Δ sdhA/pVK9 strain as the control, succinic acid-producing ability of the 2256 Δ sdhA/pVK9::*PmsrA-ynfM* strain, in which the *ynfM* gene was enhanced, was improved. These results revealed that, by amplifying the *ynfM* gene in coryneform bacteria, succinic acid-producing ability is improved.

[0230]

[Table 9]

Table 9: Influence of *ynfM* gene amplification on succinic acid production in coryneform bacterium

	Accumulation amount of succinate (g/L)±S.E.	Yield of succinate (%)±S.E.
2256 Δ sdhA/pVK9	7.8±0.43	17.3±0.89
2256 Δ sdhA/pVK9:: <i>PmsrA-ynfM</i>	12.7±0.19	22±0.33

[0231]

<Example 7> Evaluation of effect of *yjjPB* gene amplification on succinic acid production by *P. ananatis* under anaerobic condition

In this example, effect of *yjjPB* gene amplification on succinic acid production under an anaerobic condition was evaluated by using a succinic acid-producing strain derived from the *P. ananatis* SC17(0) strain as the host.

[0232]

<7-1> Construction of *P. ananatis* FKPS13 strain

By substituting the Tet^r gene for the *budABC* genes encoding the 2,3-butanediol biosynthesis system on the genome of the *P. ananatis* SC17(0) strain, *P. ananatis* FKSP 13 strain

(SC17(0) Δ budABC::Tet^r strain) was constructed. The method is shown below.

[0233]

PCR was performed by using the primers shown as SEQ ID NOS: 81 and 82, and pMW118-attL-Tet^r-attR as the template to amplify a DNA fragment for disruption of the *budABC* genes. The obtained DNA fragment was purified by using Wizard PCR Prep DNA Purification System (Promega), and introduced into competent cells of the SC17(0)/RSFRedTER strain by electroporation. The cells were cultured for 2 hours in the SOC medium, then applied to the LB agar medium containing 25 mg/L of chloramphenicol and 12.5 mg/L of tetracycline, and cultured at 34°C for 16 hours. Colonies that appeared were purified on the same medium, and then used together with the primers shown as SEQ ID NOS: 83 and 84 to perform colony PCR (TaKaRa Speed Star (registered trademark), 92°C for 10 seconds, 56°C for 10 seconds, 72°C for 30 seconds, 40 cycles), and thereby it was confirmed that the *budABC* genes on the genome was replaced with the Tet resistance gene. The obtained strain was applied to the LB agar medium containing 10% sucrose and 1 mM IPTG to remove the RSFRedTER plasmid to obtain SC17(0) Δ budABC::Tet^r strain. This strain was designated as FKSP13.

[0234]

<7-2> Construction of *P. ananatis* YDSP24

As a strain that accumulates succinic acid in a medium under an anaerobic condition, YDSP24 strain, which was deficient in the alcohol dehydrogenase *adhE* gene and the lactate dehydrogenase *ldhE* gene, and in which the pyruvate carboxylase *pyc* gene was amplified, was constructed from the FKSP13 strain. In the construction of this strain, in order to make the λ -red

111

method and removal of the drug resistance gene easier, a plasmid RSFRedIX was constructed and introduced.

[0235]

<7-2-1> Construction of RSFRedIX

PCR was performed by using the RSFParaIX plasmid (Appl. Environ. Microbiol., 2014 Nov 21, pii:AEM.03213-14) as the template, and the primers shown as SEQ ID NOS: 85 and 86 to obtain a DNA fragment comprising the *araC* gene and the *xis-int* genes of λ -phage, in which the *xis-int* genes were ligated downstream of the ParaBAD promoter in the reverse direction with respect to the *araC* gene. This DNA fragment was purified by using Wizard PCR Prep Kit (Promega), and then digested with *NotI*. This digested fragment and a fragment obtained by digesting RSFRedTER with *NotI* were ligated by using Takara DNA Ligation Kit, and the *E. coli* DH5 α strain was transformed with the ligated fragment, and applied on the LB agar medium containing 25 mg/L of chloramphenicol to obtain a transformant. The objective plasmid RSFRedIX was obtained from the transformant. RSFRedIX carries the λ -Red genes, *gam*, *bet*, and *exo*, downstream from the Plac promoter, and carries the *int-xis* genes downstream from the ParaBAD promoter. With RSFRedIX, there can be performed incorporation by the λ -Red system based on IPTG induction, and removal of drug resistance gene based on arabinose induction.

[0236]

<7-2-2> Removal of tetracycline resistance gene from SC17(0) Δ budABC::Tet^r strain

RSFRedIX was introduced into the SC17(0) Δ budABC::Tet^r strain by electroporation, and the strain was applied to the LB agar medium containing 25 mg/L chloramphenicol, and cultured at 34°C to obtain SC17(0) Δ budABC::Tet^r/RSFRedIX strain. The

112

SC17(0) Δ budABC::Tet^r/RSFRedIX strain was purified on the LB agar medium containing 25 mg/L chloramphenicol and 10 mM arabinose to obtain a plurality of single colonies. Then, they were applied to the LB agar medium containing 12.5 mg/L of tetracycline, and cultured at 34°C for 16 hours. A strain confirmed to be a strain from which the tetracycline resistance gene was removed by confirming that it could not grow was designated as SC17(0) Δ budABC/RSFRedIX strain.

[0237]

<7-2-3> Construction of *P. ananatis*

SC17(0) Δ budABC Δ adhE::Tet^r/RSFRedIX strain

PCR was performed by using the primers shown as SEQ ID NOS: 87 and 88, and pMW118-attL-Tet^r-attR as the template to amplify a DNA fragment for disruption of the *adhE* gene. The SC17(0) Δ budABC/RSFRedIX strain was cultured overnight in the LB liquid medium, 1 mL of the culture broth was inoculated into 100 mL of the LB liquid medium containing IPTG at a final concentration of 1 mM, and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. The amplified DNA fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . The cells were cultured at 34°C for 16 hours on the LB agar medium containing 12.5 mg/L of tetracycline and 25 mg/L of chloramphenicol. A colony that appeared was purified on the same medium, and used together with the primers shown as SEQ ID NOS: 89 and 90 to perform colony

PCR, and it was thereby confirmed that the *adhE* gene on the genome had been replaced with the Tet resistance gene to obtain SC17(0), Δ budABC Δ adhE::Tet^r/RSFRedIX strain.

[0238]

<7-2-4> Construction of *P. ananatis*

SC17(0),ppc::P4071-pyc::Km^r strain and

SC17(0),ldh::P4071-pyc::Km^r strain

For deletion of the *ldhA* gene encoding the lactate dehydrogenase of the SC17(0), Δ budABC Δ adhE::Tet^r/RSFRedIX strain (corresponding to the nucleotide numbers 1621607 to 1622599 of the *P. ananatis* AJ13355 strain registered as GenBank Accession Number NC_017531.1), and for enhancement of pyruvate carboxylase of the same at the same time,

SC17(0),ppc::P4071-pyc::Km^r strain was constructed first.

Then, by introducing a λ attL-Km^r- λ attR-P4071-pyc gene fragment having sequences homologous to upstream and downstream sequences of the *ldh* gene, which was prepared by PCR by using the genomic DNA of the SC17(0),ppc::P4071-pyc::Km^r strain as the template, into the SC17(0) strain by the λ -red method, SC17(0),ldh::P4071-pyc::Km^r strain was constructed.

[0239]

Construction of SC17(0),ppc::P4071-pyc::Km^r strain

First, the *ldh* gene region on the genome of SC17(0) was replaced with a sequence containing the *pyc* gene as follows. PCR was performed by using the chromosomal DNA of the *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) 2256 strain (ATCC 13869) as the template, and the synthetic DNAs shown as SEQ ID NOS: 91 and 92 as the primers to amplify a DNA fragment containing the ORF region of the *pyc* gene. Further, PCR was performed by using a DNA fragment containing

$\lambda attL$ -Km^r- $\lambda attR$ -*Ptac* (WO2008/090770A1) as the template, and the synthetic DNAs shown as SEQ ID NOS: 93 and 94 as the primers to amplify a DNA fragment containing $\lambda attL$ -Km^r- $\lambda attR$ -*P4071*. Then, PCR was performed by using the DNA fragment containing the ORF region of the *pyc* gene and the DNA fragment containing $\lambda attL$ -Km^r- $\lambda attR$ -*P4071* as the template, as well as the synthetic DNAs shown as SEQ ID NOS: 92 and 93 as the primers to obtain a $\lambda attL$ -Km^r- $\lambda attR$ -*P4071*-*pyc* gene fragment having sequences homologous to upstream and downstream sequences of the *ppc* gene at the respective ends. The $\lambda attL$ -Km^r- $\lambda attR$ -*P4071*-*pyc* gene fragment was purified by using Wizard PCR Prep (Promega), and introduced into the SC17(0)/RSF-Red-TER strain by the aforementioned λ -red method. By selecting transformants on an LB agarose plate containing 40 mg/L of kanamycin, SC17(0),*ppc*::*P4071*-*pyc*::Km^r/RSF-Red-TER strain was obtained, in which the objective characteristic was introduced into the *ppc* gene region. The obtained strain was purified on an LB agarose plate containing 10% sucrose and 1 mM IPTG to obtain a strain in which the RSF-Red-TER plasmid was removed. The obtained strain was designated as SC17(0),*ppc*::*P4071*-*pyc*::Km^r strain.

[0240]

Construction of SC17(0),*ldh*::*P4071*-*pyc*::Km^r strain

In the SC17(0),*ppc*::*P4071*-*pyc*::Km^r strain, the *pyc* gene was inserted into the *ppc* gene region, and therefore it was deficient in the *ppc* gene. Therefore, the following operations were performed in order to change the insertion position of the *pyc* gene from the *ppc* gene region to the *ldh* gene region. PCR was performed by using extracted genome of the SC17(0),*ppc*::*P4071*-*pyc*::Km^r strain as the template, and the

primers shown as SEQ ID NOS: 95 and 96 to obtain a λ attL-Km^r- λ attR-P4071-*pyc* gene fragment having sequences homologous to upstream and downstream regions of the *ldh* gene of *P. ananatis* at the respective ends. This fragment was introduced into the SC17(0)/RSF-Red-TER strain by the aforementioned λ -red method. Transformants were selected on an LB agarose plate containing 40 mg/L of kanamycin, and the genome structures thereof were confirmed by PCR using the synthetic DNAs shown as SEQ ID NOS: 97 and 98 as the primers to obtain SC17(0),*ldh*::P4071-*pyc*::Km^r/RSFRed-TER strain, in which the objective characteristic was introduced into the *ldh* gene region. This strain was deficient in the *ldh* gene instead of the *ppc* gene. The obtained strain was purified on an L agarose plate containing 10% sucrose and 1 mM IPTG to obtain a strain in which the RSF-Red-TER plasmid was removed. The obtained strain was designated as SC17(0),*ldh*::P4071-*pyc*::Km^r strain.

[0241]

<7-2-5> Construction of *P. ananatis* YDSP24

The *ldhA* gene of the SC17(0), Δ budABC Δ adhE::Tet^r/RSFRedIX strain was deleted by the aforementioned λ -red method, and pyruvate carboxylase of the same was enhanced at the same time. The procedure is shown below.

[0242]

The genome of the SC17(0),*ldh*::P4071-*pyc*::Km^r was extracted, and introduced in an amount of 700 μ g into the SC17(0), Δ budABC Δ adhE::Tet^r/RSFRedIX strain by the electric pulse method. The cells were applied on an LB agarose plate containing 12.5 mg/L of tetracycline and 50 μ g/mL of kanamycin, and cultured at 34°C for about 16 hours. For a grown

116

tetracycline and kanamycin-resistant strain, introduction of the characteristic of P4071-*pyc*::*Km^r* in the *ldh* gene region was confirmed by PCR using the synthetic DNAs shown as SEQ ID NOS: 97 and 98 as the primers, and this strain was designated as SC17(0) Δ budABC Δ adhE::Tet^r *ldh*::P4071-*pyc*::*Km^r*/RSFRedIX strain. This strain was purified in the LB agar medium containing 25 mg/L of chloramphenicol and 10 mM arabinose to obtain a plurality of single colonies. Then, they were applied to the LB agar medium containing 12.5 mg/L of tetracycline and 40 μ g/mL of kanamycin, and cultured overnight at 34°C. A strain confirmed to be a strain in which the tetracycline resistance gene and the kanamycin resistance gene were removed by confirming that it could not grow was obtained. The obtained strain was applied to the LB agar medium containing 10% sucrose and 1 mM IPTG to remove the RSFRedIX plasmid to obtain Δ SC17(0), Δ budABC Δ adhE Δ ldhA P4071-*pyc* strain. This strain was designated as YDSP24 strain.

[0243]

<7-3> Construction of pSTV28-*yjjPB*

PCR was performed in a conventional manner by using the genomic DNA of the *E. coli* W3110 strain (ATCC 27325) as the template, and the synthetic DNAs of SEQ ID NOS: 99 and 100 as the primers to obtain a DNA fragment containing the *yjjPB* genes. The obtained DNA fragment was inserted into the pSTV28 vector treated with *Bam*HI and *Pst*I using In Fusion HD Cloning Kit (Clontech). The obtained plasmid for expression of the *E. coli yjjPB* genes was designated as pSTV28-*yjjPB*.

[0244]

<7-4> Construction of *P. ananatis* YDSP27 strain and YDSP26 strain

pSTV28-yjjPB constructed by the aforementioned method was introduced into the *P. ananatis* YDSP24 strain, to thereby construct a strain that expresses *yjjPB* derived from *E. coli*, which was designated as YDSP27. Further, pSTV28 was introduced into the *P. ananatis* YDSP24 strain, to thereby construct a vector control, which was designated as YDSP26.

[0245]

Specifically, the YDSP24 strain was cultured overnight in the LB liquid medium. Then, the culture broth (100 μ L) was inoculated into fresh LB liquid medium (4 mL), and shaking culture was performed at 34°C for 3 hours. The cells were collected, washed 3 times with 10% glycerol, and used as competent cells. pSTV28-yjjPB or pSTV28 was introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . The cells were cultured for 2 hours in the SOC medium (20 g/L of Bacto tryptone, 5 g/L of yeast extract, 0.5 g/L of NaCl, 10 g/L of glucose), then applied to the LB agar medium containing 40 mg/L of chloramphenicol, and cultured for 16 hours. As a result, a transformant showing chloramphenicol resistance was obtained from each medium, and thus

SC17(0) Δ budABC Δ adhE Δ ldhAp4071-pyc/pSTV28-yjjPB strain (YDSP27 strain), and

SC17(0) Δ budABC Δ adhE Δ ldhAp4071-pyc/pSTV28 strain (YDSP26 strain) were obtained.

[0246]

<7-5> Evaluation of succinic acid-producing ability under anaerobic condition

118

Then, succinic acid-producing abilities of these strains were evaluated. These strains were each cultured overnight at 34°C on an LBGM9 agar medium plate containing 25 mg/L of chloramphenicol. The obtained cells were washed three times with a 0.85% NaCl aqueous solution to prepare a cell suspension showing an OD600 of 100. This cell suspension (0.1 ml) was inoculated into 1.3 mL of a succinic acid production medium contained in a 1.5 ml-volume tube (Eppendorf tube), and shaking culture was performed at 34° for 48 hours on an Eppendorf tube shaker (Thermomixer Comfort, Eppendorf) at 1400 rpm. The composition of the succinic acid production medium is shown below.

[0247]

[Composition of succinic acid production medium]

[Group A]

Glucose	20 g/L
MgSO ₄ · 7H ₂ O	1.0 g/L

[Group B]

(NH ₄) ₂ SO ₄	1.0 g/L
KH ₂ PO ₄	1.0 g/L
Yeast extract	2.0 g/L
FeSO ₄ · 7H ₂ O	0.01 g/L
MnSO ₄ · 5H ₂ O	0.01 g/L
Biotin	0.001 g/L

(pH was not adjusted)

[Group C]

CaCO ₃	50 g/L
-------------------	--------

The components of the groups A and B were each sterilized in an autoclave at 115°C for 10 minute, the component of Group C was sterilized with hot air at 180°C for 3 hours, then they

were mixed, and chloramphenicol was added to the mixture at 25 mg/L.

[0248]

The results are shown in Table 10. As compared with the YDSP26 strain as the control, the succinic acid carbon yield increased in the YDSP27 strain, in which the *yjjPB* genes of *E. coli* were expressed. These results revealed that, by amplifying the *yjjPB* genes in *P. ananatis*, succinic acid carbon yield thereof is improved.

[0249]

[Table 10]

Table 10: Influence of *yjjPB* gene amplification in *P. ananatis* on succinic acid production under anaerobic condition

	Succinic acid carbon yield (%)
SC17(0) Δ budABC Δ adhE Δ ldhAp4071-pyc/pSTV28	34.7 $\pm$ 2.22
SC17(0) Δ budABC Δ adhE Δ ldhAp4071-pyc/pSTV28- <i>yjjPB</i>	38.3 $\pm$ 2.36

[0250]

<Example 8> Evaluation of effect of *yjjPB* amplification on succinic acid production by *E. aerogenes* under anaerobic condition

<8-1> Construction of *E. aerogenes* ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB

E. aerogenes ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB strain deficient in the *yjjPB* genes was constructed from the *E. aerogenes* ES06 Δ sdhA Δ yeeA Δ ynfM strain by the λ -red method. Specifically, PCR was performed by using the primers shown as SEQ ID NO: 132 and 133, and pMW118-attL-Km^r-attR as the template to amplify a fragment having sequences of 50 bp complementary to an

internal sequence of the N-terminus side of the *yjjP* gene or an internal sequence of the C-terminus side of the *yjjB* gene of *Enterobacter aerogenes* at the respective ends, and the kanamycin resistance gene between the *attL* and *attR* sequences of λ phage. ES06 Δ *sdhA* Δ *yeeA* Δ *ynfM*/RSFRedIX strain obtained by introducing RSFRedIX into the ES06 Δ *sdhA* Δ *yeeA* Δ *ynfM* strain by electroporation was cultured overnight in the LB liquid medium, the culture broth (1 mL) was inoculated into the LB liquid medium (100 mL) containing IPTG at a final concentration of 1 mM and 25 mg/L of chloramphenicol, and shaking culture was performed at 34°C for 3 hours. The cells were collected, then washed 3 times with 10% glycerol, and used as competent cells. The amplified PCR fragment was purified by using Wizard PCR Prep (Promega), and introduced into the competent cells by electroporation. The electroporation was performed by using GENE PULSER II (BioRad) under the conditions of an electric field intensity of 20 kV/cm, capacitor capacity of 25 μ F, and resistance of 200 Ω . By selection on the LB agar medium containing 40 mg/L of kanamycin, ES06 Δ *sdhA* Δ *yeeA* Δ *ynfM* Δ *yjjPB::Km*/RSFRedIX strain was obtained. This strain was purified on the LB agar medium containing 25 mg/L of chloramphenicol and 10 mM arabinose to obtain a plurality of single colonies. Then, they were applied to the LB agar medium containing 40 μ g/mL of kanamycin, and cultured overnight at 34°C. A strain confirmed to be a strain in which the kanamycin resistance gene was removed by confirming that it could not grow was obtained. The obtained strain was applied to the LB agar medium containing 10% sucrose and 1 mM IPTG to remove the RSFRedIX plasmid to obtain ES06 Δ *sdhA* Δ *yeeA* Δ *ynfM* Δ *yjjPB* strain.

[0251]

<8-2> Construction of *E. aerogenes* strain for evaluation

The pSTV28 vector or the pSTV28-AeyjjPB plasmid was introduced into the ES06 Δ sdhA Δ yeeA Δ ynfM strain and the ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB strain by an electroporation, the resulting strains were subjected to selection on the LB agar medium containing 25 mg/L of chloramphenicol, and the selected strains were purified on the LBGM9 agar medium to obtain ES06 Δ sdhA Δ yeeA Δ ynfM/pSTV28 strain as a control strain, ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB/pSTV28 strain as a *yjjPB*-deficient strain, ES06 Δ sdhA Δ yeeA Δ ynfM/pSTV28-AeyjjPB strain and ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB/pSTV28-AeyjjPB strain as dicarboxylic acid efflux carrier gene-amplified strains.

[0252]

<8-3> Evaluation of succinic acid-producing ability

Then, succinic acid-producing abilities of these strains were evaluated. These strains were each cultured overnight at 34°C on an LBGM9 agar medium plate containing 25 mg/L of chloramphenicol. Then, the plate was put into AnaeroPack (Mitsubishi Gas Chemical, product number A-04), and culture was continued at 37°C for 2 hours under an anaerobic condition, and the obtained cells were used as seed cells. The seed cells on one plate were once suspended in 0.7 ml of physiological saline for washing, and re-suspended in physiological saline at an OD of 100 at 620 nm. This cell suspension was put into a 1.5 ml-volume Eppendorf tube in a volume of 300 μ l together with 900 μ l of a succinic acid production medium (drug free), the tube was sealed with a stopper, and the cells were cultured at 34°C for 24 hours with shaking at 1200 rpm on an Eppendorf tube shaker. The composition of the succinic acid production medium

122

is shown below.

[0253]

[Composition of succinic acid production medium]

[Group A]

$(\text{NH}_4)_2\text{SO}_4$	1 g/L
KH_2PO_4	1 g/L
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10 mg/L
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	10 mg/L
Yeast extract	2 g/L
(adjusted to pH 7.0 with KOH)	

[Group B]

Glucose	40 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1 g/L

The components of the groups A and B were each sterilized in an autoclave at 115°C for 10 minute, and cooled, and then CaCO_3 defined in Japanese Pharmacopoeia, which was sterilized with hot air at 180°C for 3 hours or longer, and left to cool, was added at 50 g/L.

[0254]

The results are shown in Table 11. As compared with the ES06 Δ sdhA Δ yeeA Δ ynfM/pSTV28 strain as the control, the succinic acid yield increased in the ES06 Δ sdhA Δ yeeA Δ ynfM/pSTV28-AeyjjPB strain, in which the *yjjPB* genes of *E. aerogenes* were expressed. In the ES06 Δ sdhA Δ yeeA Δ ynfM Δ yjjPB/pSTV28 strain deficient in the *yjjPB* genes, the succinic acid yield decreased by about 5% as compared with the control strain, but by introducing pSTV28-AeyjjPB into this strain, the succinic acid yield was recovered to a level equivalent to that of the ES06 Δ sdhA Δ yeeA Δ ynfM/pSTV28-AeyjjPB strain. These results

revealed that amplification of the *yjjPB* genes improves yield of succinic acid in *E. aerogenes* even under an anaerobic condition.

[0255]

[Table 11]

Table 11: Influence of *yjjPB* gene amplification in *E. aerogenes* on succinic acid production under anaerobic condition

	OD _{620nm}	Consumed glucose (g/L)	Succinate (g/L)	Fumarate (g/L)	Malate (g/L)	Acetate (g/L)	Succinate yield (%)
ES06ΔsdhAΔyeeAΔynfM /pSTV28	27±0.87	11±0.24	5.7±0.15	0.63±0.026	0.21±0.029	0.72±0.021	53±0.95
ES06ΔsdhAΔyeeAΔynfM /pSTV28-AeyjjPB	29±0.62	9.6±0.20	5.8±0.082	1.3±0.018	0.34±0.019	0.43±0.040	60±0.60
ES06ΔsdhAΔyeeAΔynfM ΔyjjPB/pSTV28	23±1.6	9.1±0.50	4.3±0.061	0.60±0.010	N.D.	0.56±0.012	48±2.1
ES06ΔsdhAΔyeeAΔynfM ΔyjjPB/pSTV28-AeyjjPB	27±1.8	9.3±0.51	5.4±0.30	1.3±0.015	0.33±0.034	0.42±0.046	57±2.6

Industrial Applicability

[0256]

According to the present invention, dicarboxylic acid-producing ability of bacteria can be improved, and dicarboxylic acids can be efficiently produced.

[0257]

<Explanation of Sequence Listing>

SEQ ID NO: 1: Nucleotide sequence of *yeeA* gene of *E. coli* MG1655

SEQ ID NO: 2: Amino acid sequence of YeeA protein of *E. coli* MG1655

SEQ ID NO: 3: Nucleotide sequence of *yeeA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 4: Amino acid sequence of YeeA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 5: Nucleotide sequence of *yeeA* gene of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 6: Amino acid sequence of YeeA protein of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 7: Nucleotide sequence of *ynfM* gene of *E. coli* MG1655

SEQ ID NO: 8: Amino acid sequence of YnfM protein of *E. coli* MG1655

SEQ ID NO: 9: Nucleotide sequence of *ynfM* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 10: Amino acid sequence of YnfM protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 11: Nucleotide sequence of *ynfM* gene of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 12: Amino acid sequence of YnfM protein of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 13: Nucleotide sequence of *ynfM* gene of *Corynebacterium glutamicum* ATCC 13032

SEQ ID NO: 14: Amino acid sequence of YnfM protein of *Corynebacterium glutamicum* ATCC 13032

SEQ ID NO: 15: Nucleotide sequence of *ynfM* gene of *Corynebacterium glutamicum* ATCC 13869

SEQ ID NO: 16: Amino acid sequence of YnfM protein of *Corynebacterium glutamicum* ATCC 13869

SEQ ID NO: 17: Nucleotide sequence of *yjjP* gene of *E. coli* MG1655

SEQ ID NO: 18: Amino acid sequence of YjjP protein of *E. coli* MG1655

SEQ ID NO: 19: Nucleotide sequence of *yjjP* gene of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 20: Amino acid sequence of YjjP protein of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 21: Nucleotide sequence of *yjjB* gene of *E. coli* MG1655

SEQ ID NO: 22: Amino acid sequence of YjjB protein of *E. coli*

125

MG1655

SEQ ID NO: 23: Nucleotide sequence of *yjjB* gene of *Enterobacter aerogenes* AJ110637

SEQ ID NO: 24: Amino acid sequence of YjjB protein of *Enterobacter aerogenes* AJ110637

SEQ ID NOS: 25 to 48: Primers

SEQ ID NO: 49: Nucleotide sequence of CAD gene of *Aspergillus terreus* optimized for codon usage of *E. coli*

SEQ ID NO: 50: Nucleotide sequence of *pckA* gene of *Actinobacillus succinogenes* 130Z

SEQ ID NO: 51: Amino acid sequence of PckA protein of *Actinobacillus succinogenes* 130Z

SEQ ID NOS: 52 to 66: Primers

SEQ ID NO: 67: Nucleotide sequence of Ptac1000 promoter

SEQ ID NOS: 68 to 100: Primers

SEQ ID NO: 101: Nucleotide sequence of *ldhA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 102: Amino acid sequence of LdhA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 103: Nucleotide sequence of *adhE* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 104: Amino acid sequence of AdhE protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 105: Nucleotide sequence of *budB* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 106: Amino acid sequence of BudB protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 107: Nucleotide sequence of *budA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 108: Amino acid sequence of BudA protein of *Pantoea*

ananatis AJ13355

SEQ ID NO: 109: Nucleotide sequence of *budC* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 110: Amino acid sequence of BudC protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 111: Nucleotide sequence of *sdhA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 112: Amino acid sequence of SdhA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 113: Nucleotide sequence of *mdh* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 114: Amino acid sequence of Mdh protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 115: Nucleotide sequence of *mgo1* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 116: Amino acid sequence of Mgo1 protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 117: Nucleotide sequence of *mgo2* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 118: Amino acid sequence of Mgo2 protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 119: Nucleotide sequence of *sfcA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 120: Amino acid sequence of SfcA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 121: Nucleotide sequence of *maeB* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 122: Amino acid sequence of MaeB protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 123: Nucleotide sequence of *aceB* gene of *Pantoea*

127

ananatis AJ13355

SEQ ID NO: 124: Amino acid sequence of AceB protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 125: Nucleotide sequence of *aceA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 126: Amino acid sequence of AceA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 127: Nucleotide sequence of *aceK* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 128: Amino acid sequence of AceK protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 129: Nucleotide sequence of *sucA* gene of *Pantoea ananatis* AJ13355

SEQ ID NO: 130: Amino acid sequence of SucA protein of *Pantoea ananatis* AJ13355

SEQ ID NO: 131: Amino acid sequence of CAD of *Aspergillus terreus*

SEQ ID NOS: 132 and 133: Primers

128

Claims

1. A method for producing a dicarboxylic acid, the method comprising:

culturing a bacterium having a dicarboxylic acid-producing ability in a medium to produce and accumulate the dicarboxylic acid in the medium; and

collecting the dicarboxylic acid from the medium, wherein the bacterium has been modified so that the expression of one or more genes selected from *yeeA* gene, *ynfM* gene, *yjjP* gene, and *yjjB* gene is increased.

2. The method according to claim 1, wherein the expression of the gene(s) is increased by increasing the copy number of the gene(s), and/or by modifying an expression control sequence of the gene(s).

3. The method according to claim 1 or 2, wherein the *yeeA* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, or 6;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, or 6 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 2, 4, or 6, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, or 5;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 1, 3, or 5, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

4. The method according to any one of claims 1 to 3, wherein the *ynfM* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 8, 10, 12, 14, or 16;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 8, 10, 12, 14, or 16 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 8, 10, 12, 14, or 16, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 7, 9, 11, 13, or 15;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 7, 9, 11, 13, or 15, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

5. The method according to any one of claims 1 to 4, wherein

130

the *yjjP* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 18 or 20;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 18 or 20 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 18 or 20, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 17 or 19;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 17 or 19, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

6. The method according to any one of claims 1 to 5, wherein the *yjjB* gene is a DNA selected from the group consisting of those defined in the following (A) to (E):

(A) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 22 or 24;

(B) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 22 or 24 but including substitution, deletion, insertion, or addition of one or several amino acid residues, and having a dicarboxylic acid-secreting activity;

(C) a DNA encoding a protein comprising an amino acid sequence

showing an identity of 90% or more to the amino acid sequence of SEQ ID NO: 22 or 24, and having a dicarboxylic acid-secreting activity;

(D) a DNA comprising the nucleotide sequence of SEQ ID NO: 21 or 23;

(E) a DNA hybridizable under stringent conditions with a sequence complementary to the nucleotide sequence of SEQ ID NO: 21 or 23, or a probe that can be prepared from such a complementary sequence, and encoding a protein having a dicarboxylic acid-secreting activity.

7. The method according to any one of claims 1 to 6, wherein the bacterium is a bacterium belonging to the family *Enterobacteriaceae*, or a coryneform bacterium.

8. The method according to claim 7, wherein the bacterium belonging to the family *Enterobacteriaceae* is a *Pantoea* bacterium or an *Enterobacter* bacterium.

9. The method according to claim 8, wherein the bacterium belonging to the family *Enterobacteriaceae* is *Pantoea ananatis* or *Enterobacter aerogenes*.

10. The method according to claim 7, wherein the coryneform bacterium is a *Corynebacterium* bacterium.

11. The method according to claim 10, wherein the coryneform bacterium is *Corynebacterium glutamicum*.

12. The method according to any one of claims 1 to 11, wherein

132

the dicarboxylic acid consists of one or more dicarboxylic acids selected from the group consisting of α -ketoglutaric acid, malic acid, fumaric acid, succinic acid, and itaconic acid.

1/1

[Fig. 1]

